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(54) Title: TISSUE SPECIFIC EXPRESSION OF RETINOBLASTOMA PROTEIN

(57) Abstract

Fusions of the transcription factor E2F and the retinoblastoma protein RB are provided, along with methods of treatment of hyperproliferative diseases.

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TISSUE SPECIFIC EXPRESSION OF RETINOBLASTOMA PROTEIN

BACKGROUND OF THE INVENTION

Both the retinoblastoma gene (RB) and transcription factor E2F play a critical role in cell growth control (for a review, see Adams, P. & Kaelin, W. Seminars in Cancer Biology 6:99-108 (1995)). The RB locus is frequently inactivated in a variety of human tumor cells. Reintroduction of a wild-type RB gene (e.g., Bookstein et al. Science 247:712-715 (1990)) or RB protein (pRB) (e.g., Antelman et al. Oncogene 10:697-704(1995)) into RBneg/RBmut cells can suppress growth in culture and tumorigenicity *in vivo*.

While E2F serves to activate transcription of S-phase genes, its activity is kept in check by RB. RB arrests cells by blocking exit from G into S-phase (for example, Dowdy et al. Cell 73:499-511 (1993)) but the precise pathway of the arrest remains unclear.

Although E2F forms complexes with RB, complex formation is more efficient if an E2F-related protein, DP-1, is present. E2F-1 and DP-1 form stable heterodimers which bind to DNA (for example, Qin et al. Genes and Dev. 6-:953-964 (1992)). DP-1-E2F complexes serve to cooperatively activate transcription of E2F-dependent genes. Such transcription can be repressed by pRB in the same manner as E2F-1 or DP-1 activated transcription.

Transcriptional repression of genes by RB in some instances can be achieved by tethering pRB to a promoter. For example, GAL4-pRB fusions bind to GAL4 DNA binding domains and repress transcription from p53, Sp-1 or AP-1 elements (Adnane, et al. J. Biol. Chem. 270:8837-8843 (1995); Weintraub, et al. Nature 358:259-261 (1995)). Sellers, et al. (Proc. Natl. Acad. Sci. 92:11544-11548 (1995)) disclosed fusions of amino

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acid residues 1-368 of E2F with amino acids 379-792 or 379-928 of RB.

Chang, et al. (Science **267:518-521** (1995)) disclosed the use of a replication-defective adenovirus-RB construct in the reduction of neointima formation in two animal models of restenosis, a hyperproliferative disorders.

SUMMARY OF THE INVENTION

The instant invention provides the surprising result that a fusion of an E2F polypeptide with an RB polypeptide is more efficient in repressing transcription of the E2F promoter than RB alone, and that such fusions can cause cell cycle arrest in a variety of cell types. Such fusions can thus address the urgent need for therapy of hyperproliferative disorders, including cancer.

One aspect of the invention is a polypeptide comprising a fusion of a transcription factor, the transcription factor comprising a DNA binding domain, and a retinoblastoma (RB) polypeptide, the RB polypeptide comprising a growth suppression domain. Another aspect of the invention is DNA encoding such a fusion polypeptide. The DNA can be inserted in an adenovirus vector.

In some embodiments of the invention, the transcription factor is E2F. The cyclin A binding domain of the E2F can be deleted or nonfunctional. The E2F can comprise amino acid residues about 95 to about 194 or about 95 to about 286 in some embodiments.

The retinoblastoma polypeptide can be wild-type RB, RB56, or a variant or fragment thereof. In some embodiments, the retinoblastoma polypeptide comprises amino acid residues of about 379 to about 928. Preferred amino acid substitutions of the RB polypeptide include residues 2, 608, 788, 807, and 811.

Another aspect of the invention is an expression vector comprising DNA encoding a polypeptide, the polypeptide comprising a fusion of a transcription factor, the transcription factor comprising a DNA binding domain, and a retinoblastoma (RB) polypeptide, the RB polypeptide comprising

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a growth suppression domain. In some embodiments a tissue-specific promoter is operatively linked to DNA encoding the fusion polypeptide. The tissue-specific promoter can be a smooth muscle alpha actin promoter.

Another aspect of the invention is a method for treatment of hyperproliferative disorders comprising administering to a patient a therapeutically effective dose of an E2F-RB fusion polypeptide. The hyperproliferative disorder can be cancer. In some embodiments the hyperproliferative disorder is restenosis. The fusion polypeptide and nucleic acid encoding the fusion polypeptide can be used to coat devices used for angioplasty.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A depicts the predicted amino acid sequence of E2F.

Figure 1B depicts the nucleotide sequence of transcription factor E2F.

Figure 2A depicts the nucleotide sequence of pRB as disclosed by Lee, et al. (Nature **329:642-645** (1987).

Figure 2B depicts the predicted amino acid sequence of pRB.

Figure 3 is a diagrammatic representation of pCTM.

Figure 4 depicts the nucleotide sequence of plasmid pCTM.

Figure 5 is a diagrammatic representation of pCTMI.

Figure 6 depicts the nucleotide sequence of pCTMI.

Figure 7 is a diagrammatic representation of plasmid pCTMIE.

Figure 8 depicts the nucleotide sequence of pCTMIE.

Figure 9 is a diagram depicting E2F-RB fusion constructs used in the examples. All E2F constructs commenced at amino acid 95 and lacked part of the cyclin A binding domain. E2F-437 contained the DNA binding domain (black), heterodimerization domain (white), and the transactivation domain (stippled). E2F-194 contained solely the DNA binding domain. E2F-286 contained the DNA binding domain and the DP-1 heterodimerization domain. To generate E2F-194-RB56-5s and

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E2F-286-RB56-5s, the E2F constructs were fused in-frame to codon 379 of RB. C706F is an inactivating point mutation.

Figure 10 is a diagram depicting transcriptional repression by E2F-RB fusion constructs.

Figure 11 (A-D) depicts expression of E2F-RB fusion proteins in mammalian cell lines. Extracts were prepared from cells used in E2-CAT reporter assays or in FACS assays and analyzed with an anti-RB monoclonal antibody. In panel A, the results are shown from C33A cells transfected with (3) RB56-H209, (4) RB56 wild-type, (5) RB56-5s, (6) E2F286-5s, (7) E2F194-5s, (8) E2F194, (9) E2F286, (10) E2F437. Lane (1) is an RB56 protein standard. Lane (2) is a mock transfection. In panel B, results are shown for transfection of Saos-2 cells with (1) RB56, (2,3) E2F194-5s, and (4,5) E2F286-5s. In panel C, results are shown for transfection of 5637 cells with (2,3) RB56 wild-type, (4,5) RB56-5s; (6,7) E2F194-5s; (7,8) E2F286-5S. Lane (1) is an RB56 protein standard. In panel D, results are shown for NIH-3T3 transfected (3) RB56, (4) E2F286-5s, (5) E2F194-5s. Lane (1) is an RB56 standard; lane (2) is an RB110 standard.

Figure 12 depicts histogram analyses of flow cytometry of RB-expressing NIH-3T3 cells.

Figure 13, panel A, depicts a comparison of the effects of a CMV-driven recombinant adenovirus (ACN56) with two isolates of a human smooth muscle alpha actin-driven E2F-p56 fusion construct consisting of amino acids 95 through 286 of E2F linked directly and in-frame to p56 (amino acids 379-928 of RB cDNA), vs. a control virus (ACN) in a ^{3}H-thymidine uptake assay in the rat smooth muscle cell line A7R5. Panel (B) depicts the effects of the same constructs in the rat smooth muscle cell line A10.

Figure 14 depicts a comparison of the effects of the viruses described in Fig. 13 in non-muscle cells. Panel (A) depicts results in the breast carcinoma cell line MDA MB468. Panel (B) depicts results in the non-small cell lung cell carcinoma line H358.

Figure 15, top panel, depicts the relative infectivity by adenovirus of different cell lines as judged by

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the level of β-galactosidase (β-gal) staining following infection with equal amounts of a recombinant adenovirus expressing β-gal driven by a CMV promoter. H358 is non-small lung cell carcinoma cell line; MB468 is a breast carcinoma cell line; A7R5 and A10 are smooth muscle cell lines. The lower portion of the figure depicts the relative levels of p56 protein expressed in the same cells when infected with the recombinant adenovirus ACN56, in which the p56 cDNA is driven by the non-tissue specific CMV promoter.

Figure 16 depicts relative protein levels in cells infected with the smooth muscle alpha actin promoter-driven E2F-p56 fusion construct (ASN286-56). UN denoted uninfected; 50, 100, 250, and 500 refer to multiplicities of infection (MOI).

Figure 17 is a bar graph depicting the ratio of intima to media area (as a measurement of the inhibition of neointima formation) from cross-sections (n=9) of rat carotid arteries which were injured and treated with recombinant adenoviruses expressing either β-gal, RB (ACNRB) or p56 (ACN56), all under the control of the CMV promoter.

Figure 18 is a series of three photographs depicting restenosis in a rat angioplasty model. The panel on the left depicts data from a normal animal; the central panel depicts data from an animal injured and then treated with a β-gal expressing recombinant virus; the panel on the right depicts data from an animal injured and then treated with a recombinant adenovirus expressing p56 (ACN56).

Figure 19 depicts tissue-specificity of the smooth muscle alpha actin promoter, as demonstrated by its selective ability to express the β-gal transgene in muscle cells but not non-muscle cells. The panels on the left compare β-gal expression in the breast cell carcinoma line MB468 infected with either an MOI=1 with a CMV-driven β-gal (ACNBGAL) vs an MOI= 100 with the smooth muscle promoter construct (ASNBGAL). The panels on the right show β-gal expression of the rat smooth muscle cell line A7R5 infected with either an MOI=1 of ACNBGAL or an MOI=50 of ASNBGAL. Expression from ASNBGAL is seen in the muscle cell line, but is absent in the non-muscle

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cell line, despite the higher degree of infectivity of the cells.

Figure 20 depicts the ability of recombinant adenovirus expressing RB to transduce rat carotid arteries. recombinant adenovirus-treated arteries (1X 10^9 pfu) were harvested two days following balloon injury and infection. Cross sections were fixed and an RB specific antibody was used to detect the presence of RB protein in the tissue. The control virus used was ACN. RB protein staining was evident in the ACNRB treated sample, especially at higher magnifications.

Figure 21 depicts a comparison of the effects of a CMV-driven p56 recombinant adenovirus (ACN56E4) vs a human smooth muscle alpha-actin promoter-driven E2F-p56 fusion construct (ASN286-56) vs control adenoviral constructs containing either the CMV or smooth muscle alpha-actin promoters without a downstream transgene (ACNE3 or ASBE3-2 isolates shown, respectively). Assays were ^{3}H-thymidine uptake either in a smooth muscle cell line (A7R5) or a non-muscle cell line (MDA-MB468, breast carcinoma). Results demonstrated muscle tissue specificity using the smooth muscle alpha-actin promoter and specific inhibition by both the p56 and E2F-p56 transgenes relative to their respective controls.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The instant invention provides RB fusion constructs including fusion polypeptides and vectors encoding them, and methods for the use of such constructs in the treatment of hyperproliferative diseases. In some preferred embodiments of the invention, an RB polypeptide is fused to an E2F polypeptide. Any E2F species can be used, typically E2F-1, -2, -3, -3, or -5 (see, e.g., Wu et al. Mol Cell. Biol. 15:2536-2546 (1995); Ivey-Hoyle et al. Mol. Cell. Biol. 13:7802 (1993); Vairo et al. Genes and Dev. 9:869 (1995); Beijersbergen et al. Genes and Dev. 8:2680 (1994)); Ginsberg et al. Genes and Dev. 8:2665 (1994); Buck et al. Oncogene 11:31 (1995)), more typically E2F-1. Typically, the EF2

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polypeptide comprises at least the DNA binding domain of E2F, and may optionally include the cyclin A binding domain, the heterodimerization domain, and/or the transactivation domain. Preferably, the cyclin A binding domain is not functional. The nucleotide and amino acid sequence of E2F referred to herein are those of Genbank HUME2F, shown in Figure 1A and 1B. Nucleic acid, preferably DNA, encoding such an EF2 polypeptide is fused in reading frame to an RB polypeptide. The RB polypeptide can be any RB polypeptide, including conservative amino acid variants, allelic variants, amino acid substitution, deletion, or insertion mutants, or fragments thereof. Preferably, the growth suppression domain, i.e., amino acids residues 379-928, of the RB polypeptide is functional (Hiebert, et al. MCB **13:3384-3391** (1993); Qin, et al. Genes and Dev. **6:953-964** (1992)). In some embodiments, wild-type pRB110 is used. More preferably, a truncated version of RB, RB56, is used. RB56 comprises amino acid residues 379-928 of pRB110 (Hiebert, et al. MCB **13:3384-3391** (1993); Qin, et al. Genes and Dev. **6:953-964** (1992)). In some embodiments, amino acid variants of RB at positions 2, 608, 612, 788, 807, or 811, are used singly or in combination. The variant RB56-5s comprises wild-type RB56 having alanine substitutions at 608, 612, 788, 807, and 811. Numbering of RB amino acids and nucleotides is according to the RB sequence disclosed by Lee, et al. (Nature **329:642-645** (1987)), hereby incorporated by reference in its entirety for all purposes. (Figure 2).

Nucleic acids encoding the polypeptides of the invention can be DNA or RNA. The phrase "nucleic acid sequence encoding" refers to a nucleic acid which directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The nucleic acid sequences include both the full length nucleic acid sequences as well as non-full length sequences derived from the full length protein. It is further understood that the sequence includes the degenerate codons of

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the native sequence or sequences which may be introduced to provide codon preference in a specific host cell.

The term "vector" as used herein refers to viral expression systems, autonomous self-replicating circular DNA (plasmids), and includes both expression and nonexpression plasmids. Where a recombinant microorganism or cell culture is described as hosting an "expression vector," this includes both extrachromosomal circular DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome. A vector contains multiple genetic elements positionally and sequentially oriented, i.e., operatively linked with other necessary elements such that nucleic acid in the vector encoding the constructs of the invention can be transcribed, and when necessary, translated in transfected cells.

The term "gene" as used herein is intended to refer to a nucleic acid sequence which encodes a polypeptide. This definition includes various sequence polymorphisms, mutations, and/or sequence variants wherein such alterations do not affect the function of the gene product. The term "gene" is intended to include not only coding sequences but also regulatory regions such as promoters, enhancers, and termination regions. The term further includes all introns and other DNA sequences spliced from the mRNA transcript, along with variants resulting from alternative splice sites.

The term "plasmid" refers to an autonomous circular DNA molecule capable of replication in a cell, and includes both the expression and nonexpression types. Where a recombinant microorganism or cell culture is described as hosting an "expression plasmid", this includes both extrachromosomal circular DNA molecules and DNA that has been incorporated into the host chromosome(s). Where a plasmid is being maintained by a host cell, the plasmid is either being stably replicated by the cells during mitosis as an autonomous structure or is incorporated within the host's genome.

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The phrase "recombinant protein" or "recombinantly produced protein" refers to a peptide or protein produced using non-native cells that do not have an endogenous copy of DNA able to express the protein. The cells produce the protein because they have been genetically altered by the introduction of the appropriate nucleic acid sequence. The recombinant protein will not be found in association with proteins and other subcellular components normally associated with the cells producing the protein. The terms "protein" and "polypeptide" are used interchangeably herein.

In general, a construct of the invention is provided in an expression vector comprising the following elements linked sequentially at appropriate distances for functional expression: a tissue-specific promoter, an initiation site for transcription, a 3' untranslated region, a 5' mRNA leader sequence, a nucleic acid sequence encoding a polypeptide of the invention, and a polyadenylation signal. Such linkage is termed "operatively linked." Enhancer sequences and other sequences aiding expression and/or secretion can also be included in the expression vector. Additional genes, such as those encoding drug resistance, can be included to allow selection or screening for the presence of the recombinant vector. Such additional genes can include, for example, genes encoding neomycin resistance, multi-drug resistance, thymidine kinase, beta-galactosidase, dihydrofolate reductase (DHFR), and chloramphenicol acetyl transferase.

In the instant invention, tissue-specific expression of the RB constructs of the invention is preferably accomplished by the use of a promoter preferentially used by a tissue of interest. Examples of tissue-specific promoters include the promoter for creatine kinase, which has been used to direct the expression of dystrophin cDNA expression in muscle and cardiac tissue (Cox, et al. Nature **364:725-729** (1993)) and immunoglobulin heavy or light chain promoters for the expression of suicide genes in B cells (Maxwell, et al. Cancer Res. **51:4299-4304** (1991)). An endothelial cell-specific regulatory region has also been characterized (Jahroudi, et al. Mol. Cell. Biol. **14:999-1008** (1994)).

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Amphotrophic retroviral vectors have been constructed carrying a herpes simplex virus thymidine kinase gene under the control of either the albumin or alpha-fetoprotein promoters (Huber, et al. Proc. Natl. Acad. Sci. U.S.A. **88:8039-8043** (1991)) to target cells of liver lineage and hepatoma cells, respectively. Such tissue specific promoters can be used in retroviral vectors (Hartzoglou, et al. J. Biol. Chem. **265:17285-17293** (1990)) and adenovirus vectors (Friedman, et al. Mol. Cell. Biol. **6:3791-3797** (1986); Wills et al. Cancer Gene Therapy 3:191-197 (1995)) and still retain their tissue specificity.

In the instant invention, a preferred promoter for tissue-specific expression of exogenous genes is the human smooth muscle alpha-actin promoter. Reddy, et al. (J. Cell Biology **265:1683-1687** (1990)) disclosed the isolation and nucleotide sequence of this promoter, while Nakano, et al. (Gene **99:285-289** (1991)) disclosed transcriptional regulatory elements in the 5' upstream and the first intron regions of the human smooth muscle (aortic type) alpha-actin gene.

Petropoulos, et al. (J. Virol. **66:3391-3397** (1992)) disclosed a comparison of expression of bacterial chloramphenicol transferase (CAT) operatively linked to either the chicken skeletal muscle alpha actin promoter or the cytoplasmic beta-actin promoter. These constructs were provided in a retroviral vector and used to infect chicken eggs.

Exemplary tissue-specific expression elements for the liver include but are not limited to HMG-CoA reductase promoter (Luskey, Mol. Cell. Biol. **7(5):1881-1893** (1987)); sterol regulatory element 1 (SRE-1; Smith et al. J. Biol. Chem. **265(4):2306-2310** (1990); phosphoenol pyruvate carboxy kinase (PEPCK) promoter (Eisenberger et al. Mol. Cell Biol. **12(3):1396-1403** (1992)); human C-reactive protein (CRP) promoter (Li et al. J. Biol. Chem. **265(7):4136-4142** (1990)); human glucokinase promoter (Tanizawa et al. Mol. Endocrinology **6(7):1070-81** (1992); cholesterol 7-alpha hydroylase (CYP-7) promoter (Lee et al. J. Biol. Chem. **269(20):14681-9** (1994)); beta-galactosidase alpha-2,6 sialyltransferase promoter

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(Svensson et al. J. Biol. Chem. 265(34):20863-8 (1990); insulin-like growth factor binding protein (IGFBP-1) promoter (Babajko et al. Biochem Biophys. Res. Comm. 196 (1):480-6 (1993)); aldolase B promoter (Bingle et al. Biochem J. 294(Pt2):473-9 (1993)); human transferrin promoter (Mendelzon et al. Nucl. Acids Res. 18(19):5717-21 (1990); collagen type I promoter (Houglum et al. J. Clin. Invest. 94(2):808-14 (1994)).

Exemplary tissue-specific expression elements for the prostate include but are not limited to the prostatic acid phosphatase (PAP) promoter (Banas et al. Biochim. Biophys. Acta. 1217(2):188-94 (1994); prostatic secretory protein of 94 (PSP 94) promoter (Nolet et al. Biochim. Biophys. ACTA 1098(2):247-9 (1991)); prostate specific antigen complex promoter (Casper et al. J. Steroid Biochem. Mol. Biol. 47 (1-6):127-35 (1993)); human glandular kallikrein gene promoter (hgt-1) (Lilja et al. World J. Urology 11(4):188-91 (1993).

Exemplary tissue-specific expression elements for gastric tissue include but are not limited to the human H^+/K^+-ATPase alpha subunit promoter (Tanura et al. FEBS Letters 298:(2-3):137-41 (1992)).

Exemplary tissue-specific expression elements for the pancreas include but are not limited to pancreatitis associated protein promoter (PAP) (Dusetti et al. J. Biol. Chem. 268(19):14470-5 (1993)); elastase 1 transcriptional enhancer (Kruse et al. Genes and Development 7(5):774-86 (1993)); pancreas specific amylase and elastase enhancer promoter (Wu et al. Mol. Cell. Biol. 11(9):4423-30 (1991); Keller et al. Genes & Dev. 4(8):1316-21 (1990)); pancreatic cholesterol esterase gene promoter (Fontaine et al. Biochemistry 30(28):7008-14 (1991)).

Exemplary tissue-specific expression elements for the endometrium include but are not limited to the uteroglobin promoter (Helftenbein et al. Annal. NY Acad. Sci. 622:69-79 (1991)).

Exemplary tissue-specific expression elements for adrenal cells include but are not limited to cholesterol side-

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chain cleavage (SCC) promoter (Rice et al. J. Biol. Chem. 265:11713-20 (1990).

Exemplary tissue-specific expression elements for the general nervous system include but are not limited to gamma-gamma enolase (neuron-specific enolase, NSE) promoter (Forss-Petter et al. Neuron 5(2):187-97 (1990)).

Exemplary tissue-specific expression elements for the brain include but are not limited to the neurofilament heavy chain (NF-H) promoter (Schwartz et al. J. Biol. Chem. 269(18):13444-50 (1994)).

Exemplary tissue-specific expression elements for lymphocytes include but are not limited to the human CGL-1/granzyme B promoter (Hanson et al. J. Biol. Chem. 266 (36):24433-8 (1991)); the terminal deoxy transferase (TdT), lambda 5, VpreB, and lck (lymphocyte specific tyrosine protein kinase p561ck) promoter (Lo et al. Mol. Cell. Biol. 11(10):5229-43 (1991)); the humans CD2 promoter and its 3'transcriptional enhancer (Lake et al. EMBO J. 9(10):3129-36 (1990)), and the human NK and T cell specific activation (NKG5) promoter (Houchins et al. Immunogenetics 37(2):102-7 (1993)).

Exemplary tissue-specific expression elements for the colon include but are not limited to pp60c-src tyrosine kinase promoter (Talamonti et al. J. Clin. Invest 91(1):53-60 (1993)); organ-specific neoantigens (OSNs), mw 40kDa (p40) promoter (Ilantzis et al. Microbiol. Immunol. 37(2):119-28 (1993)); colon specific antigen-P promoter (Sharkey et al. Cancer 73(3 supp.) 864-77 (1994)).

Exemplary tissue-specific expression elements for breast cells include but are not limited to the human alpha-lactalbumin promoter (Thean et al. British J. Cancer. 61(5):773-5 (1990)).

Other elements aiding specificity of expression in a tissue of interest can include secretion leader sequences, enhancers, nuclear localization signals, endosmolytic peptides, etc. Preferably, these elements are derived from the tissue of interest to aid specificity.

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Techniques for nucleic acid manipulation of the nucleic acid sequences of the invention such as subcloning nucleic acid sequences encoding polypeptides into expression vectors, labelling probes, DNA hybridization, and the like are described generally in Sambrook *et al.*, Molecular Cloning - A Laboratory Manual (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989), which is incorporated herein by reference. This manual is hereinafter referred to as "Sambrook *et al.*"

Once DNA encoding a sequence of interest is isolated and cloned, one can express the encoded proteins in a variety of recombinantly engineered cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of DNA encoding. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes is made here.

In brief summary, the expression of natural or synthetic nucleic acids encoding a sequence of interest will typically be achieved by operably linking the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of polynucleotide sequence of interest. To obtain high level expression of a cloned gene, it is desirable to construct expression plasmids which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. The expression vectors may also comprise generic expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the plasmid in both eukaryotes and prokaryotes, *i.e.*, shuttle vectors, and selection markers for both prokaryotic and eukaryotic systems. *See* Sambrook *et al.*

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The E2F-RB fusion constructs of the invention can be introduced into the tissue of interest in vivo or ex vivo by a variety of methods. In some embodiments of the invention, the nucleic acid, preferably DNA, is introduced to cells by such methods as microinjection, calcium phosphate precipitation, liposome fusion, or biolistics. In further embodiments, the DNA is taken up directly by the tissue of interest. In other embodiments, the constructs are packaged into a viral vector system to facilitate introduction into cells.

Viral vector systems useful in the practice of the instant invention include adenovirus, herpesvirus, adeno-associated virus, minute virus of mice (MVM), HIV, sindbis virus, and retroviruses such as Rous sarcoma virus, and MoMLV. Typically, the constructs of the instant invention are inserted into such vectors to allow packaging of the E2F-RB expression construct, typically with accompanying viral DNA, infection of a sensitive host cell, and expression of the E2F-RB gene. A particularly advantageous vector is the adenovirus vector disclosed in Wills, et al. Human Gene Therapy **5:1079-1088** (1994).

In still other embodiments of the invention, the recombinant DNA constructs of the invention are conjugated to a cell receptor ligand for facilitated uptake (e.g., invagination of coated pits and internalization of the endosome) through a DNA linking moiety (Wu, et al. J. Biol. Chem. **263:14621-14624** (1988); WO 92/06180). For example, the DNA constructs of the invention can be linked through a polylysine moiety to asialo-oromucocid, which is a ligand for the asialoglycoprotein receptor of hepatocytes.

Similarly, viral envelopes used for packaging the constructs of the invention can be modified by the addition of receptor ligands or antibodies specific for a receptor to permit receptor-mediated endocytosis into specific cells (e.g., WO 93/20221, WO 93/14188; WO 94/06923). In some embodiments of the invention, the DNA constructs of the invention are linked to viral proteins, such as adenovirus particles, to facilitate endocytosis (Curiel, et al. Proc. Natl. Acad. Sci. U.S.A. **88:8850-8854** (1991)). In other

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embodiments, molecular conjugates of the instant invention can include microtubule inhibitors (WO 94/06922); synthetic peptides mimicking influenza virus hemagglutinin (Plank, et al. J. Biol. Chem. **269:12918-12924** (1994)); and nuclear localization signals such as SV40 T antigen (WO 93/19768).

In some embodiments of the invention, the RB polypeptides of the invention are administered directly to a patient in need of treatment. A "therapeutically effective" dose is a dose of polypeptide sufficient to prevent or reduce severity of a hyperproliferative disorder. As used herein, the term "hyperproliferative cells" includes but is not limited to cells having the capacity for autonomous growth, i.e., existing and reproducing independently of normal regulatory mechanisms. Hyperproliferative diseases may be categorized as pathologic, i.e., deviating from normal cells, characterizing for constituting disease, or may be categorized as non-pathologic, i.e., deviation from normal but not associated with a disease state. Pathologic hyperproliferative cells are characteristic of the following disease states: restenosis, diabetic retinopathy, thyroid hyperplasia, Grave's disease, psoriasis, benign prostatic hypertrophy, Li-Fraumeni syndrome including breast cancer, sarcomas and other neoplasms, bladder cancer, colon cancer, lung cancer, various leukemias and lymphomas. Examples of non-pathological hyperproliferative cells are found, for instance, in mammary ductal epithelial cells during development of lactation and also in cells associated with wound repair. Pathological hyperproliferative cells characteristically exhibit loss of contact inhibition and a decline in their ability to selectively adhere which implies a further breakdown in intercellular communication. These changes include stimulation to divide and the ability to secrete proteolytic enzymes.

The constructs of the invention are useful in the therapy of various cancers and other conditions in which the administration of RB is advantageous, including but not limited to peripheral vascular diseases and diabetic retinopathy. Although any tissue can be targeted for which

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some tissue-specific expression element, such as a promoter, can be identified, of particular interest is the tissue-specific administration of an RB construct for hyperproliferative disorders such as restenosis, for which the smooth muscle actin promoter is preferable.

The compositions of the invention will be formulated for administration by manners known in the art acceptable for administration to a mammalian subject, preferably a human. In some embodiments of the invention, the compositions of the invention can be administered directly into a tissue by injection or into a blood vessel supplying the tissue of interest. In further embodiments of the invention the compositions of the invention are administered "locoregionally", i.e., intravesically, intralesionally, and/or topically. In other embodiments of the invention, the compositions of the invention are administered systemically by injection, inhalation, suppository, transdermal delivery, etc. In further embodiments of the invention, the compositions are administered through catheters or other devices to allow access to a remote tissue of interest, such as an internal organ. The compositions of the invention can also be administered in depot type devices, implants, or encapsulated formulations to allow slow or sustained release of the compositions.

The invention provides compositions for administration which comprise a solution of the compositions of the invention dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents,

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wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of the compositions of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The compositions of the invention may also be administered via liposomes. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the composition of the invention to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a desired target, such as antibody, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired composition of the invention of the invention can delivered systemically, or can be directed to a tissue of interest, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions.

Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al. Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

A liposome suspension containing a composition of the invention may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the composition of the

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invention being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more compositions of the invention of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the compositions of the invention are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of compositions of the invention are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

The constructs of the invention can additionally be delivered in a depot-type system, an encapsulated form, or an implant by techniques well-known in the art. Similarly, the constructs can be delivered via a pump to a tissue of interest.

In some embodiments of the invention, the compositions of the invention are administered ex vivo to cells or tissues explanted from a patient, then returned to

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the patient. Examples of ex vivo administration of gene therapy constructs include Arteaga et al. Cancer Research 56(5):1098-1103 (1996); Nolta et al. Proc Natl. Acad. Sci. USA 93(6):2414-9 (1996); Koc et al. Seminars in Oncology 23 (1):46-65 (1996); Raper et al. Annals of Surgery 223(2):116-26 (1996); Dalesandro et al. J. Thorac. Cardi. Surg. 11(2):416-22 (1996); and Makarov et al. Proc. Natl. Acad. Sci. USA 93(1):402-6 (1996).

In some embodiments of the invention, the constructs of the invention are administered to a cardiac artery after balloon angioplasty to prevent or reduce the severity of restenosis. The constructs of the invention can be used to coat the device used for angioplasty (see, for example, Willart, et al. Circulation 89:2190-2197 (1994); French, et al. Circulation 90:2402-2413 (1995)). In further embodiments, the fusion polypeptides of the invention can be used in the same manner.

The following examples are included for illustrative purposes and should not be considered to limit the present invention.

EXAMPLES

Example I

E2F-RB Fusions

A. Introduction

In this example, expression plasmids which encode different segments of E2F fused to RB56 polypeptide were constructed. RB56 is a subfragment of full length RB which contains the "pocket" domains necessary for growth suppression (Hiebert, et al. MCB 13:3384-3391 (1993); Qin, et al. Genes and Dev. 6:953-964 (1992)). E2F194 contains E2F amino acids 95-194. This fragment contains only the DNA binding domain of E2F. E2F286 contains the DNA binding domain and the DP-1 heterodimerization domain. Both E2F fragments lack the N-terminal cyclin A-kinase binding domain, which appears to down-regulate the DNA binding activity of E2F (Krek et al. Cell 83:1149-1158 (1995); Krek et al. Cell 78:161-172 (1994)).

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B. Construction of Vectors

Plasmid pCTM contains a CMV promoter, a tripartite adenovirus leader flanked by T7 and SP6 promoters, and a multiple cloning site with a bovine growth hormone (BGH) polyadenylation site and a SV-40 poly adenylation site downstream. A diagrammatic representation of pCTM is provided in Figure 3. The DNA sequence for pCTM is provided in Figure 4.

pCTMI was constructed from pCTM by digesting pCTM with Xho I and Not I and subcloning a 180 bp intron Xhol-Not I fragment from a pCMV-β-gal vector (Clonetech). A diagrammatic representation of pCTMI is provided in Figure 5. The DNA sequence is provided in Figure 6.

pCTMIE was constructed by amplifying the SV40 enhancer from SV40 viral DNA in a polymerase chain reaction. The amplified product was digested with BglII and inserted into BamH1-digested pCMTI and ligated in the presence of BamHI. The plasmid is depicted diagrammatically in Figure 7. The DNA sequence is provided in Figure 8.

pCTM-RB was prepared as follows. A 3.2 KB Xba I - Cla I fragment of pETRBc (Huang et al. Nature 350:160-162 (1991)) containing the full length human RB cDNA was ligated to Xba I-Cla I digested pCTM. pCTM-RB56 was prepared by ligating the digested pCTM to a 1.7 KB Xba 1 -Cla I fragment containing the coding sequence for RB56. pCTMI-RB, pCTMIE-RB, pCTMI-RB56(amino acids 381-928) and pCTMIE-RB56(amino acids 381-928) were all constructed by the same methods.

C. RB-E2F fusion Constructs

Figure 9 depicts the fusion constructs used in these studies. These E2F constructs commenced at amino acid 95 and lacked part of the cyclin A binding domain. E2F437 contained the DNA binding domain (black), heterodimerization domain (white) and transactivation domain (stippled). E2F194 contained solely the DNA binding domain. E2F286 contained the DNA binding domain and DP-1 heterodimerization domain. RB56-5s refers to an RB variant having alanine substitutions at amino acid residues 606, 612, 788, 807 and 811. In E2F194-

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RB56-5s and E2F286-RB56-5s, the E2F fragments were fused in frame to codon 379 of RB-5s. RB56-C706F contained an inactivating point mutation (Kaye et al. Proc. Natl. Acad. Sci. U.S.A. 87:6922-6926 (1990)).

pCMV-E2F194 and pCMV-E2F437 were constructed as follows. DNA encoding amino acids 95-194 of E2F (containing the DNA binding domain) or amino acids 95-437 was amplified in a polymerase chain reaction, digested with HindII, and ligated into SmaI/HindII digested pCMV-RB56 vectors. pCMVE2F286 was constructed by digesting pCMV-E2F437 with AflII, treating the ends with DNA pol I (Klenow fragment) and religating in the presence of AflII. The blunt end ligation created a stop codon at position 287. pCMV-E2F286-5s was constructed by ligating AflII (blunt)/HindIII digested pE2F437 to a Sal I (blunt)-HindIII fragment containing the RB56-5s coding sequence. pCTMIE-E2F194-5s and pCTMIE-E2F286-RB5s were constructed by ligating EcoRI-EcoRV digested pCTMIE (4.2 KB) to HindIII (blunt)-EcoRI fragments from either pCMV-E2F194-RB5s or pCMV-E2F286-RB5s.

D. Promoter Repression

To measure the effect of the E2F-RB fusion proteins, cervical carcinoma cell line C33A (ATCC # HTB-31) was transfected with equivalent amounts of E2F194-RB56 or E2F RB56 with an E2-CAT reporter plasmid (See, e.g., Weintraub et al. Nature 358:259-261 (1992)).

In the C33A assay, 250,000 C33A cells were seeded into each of well of 6-well tissue culture plates and allowed to adhere overnight. 5 μg each of pCMV-RB56, pCMV-E2F RB56, or pCMV-E2F plasmid were cotransfected (calcium phosphate method, MBS transfection kit, Stratagene) with 5 μg of indicated reporter construct E2-CAT or SVCAT) and 2.5 μg β-gal plasmid (pCMV-β, Clontech) per well into duplicate wells. Cells were harvested 72 hour after transfection and extracts were prepared.

In the 5637 assay, 250,000 5637 cells were seeded as described above. 1 μg each of RB or E2F-RB fusion plasmid, E2-CAT or SV-CAT reporter plasmid and pCMV-β-galactosidase

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were cotransfected using the lipofectin reagent (BRL, Bethesda, Maryland) according to the manufacturer's instructions.

CAT assays were performed using either 20 μL (C33A) or 50 μL (5637) of cell extract (Gorman et al. Mol. Cell. Biol. 2:1044 (1982)). TLCs were analyzed on a Phosphoimager SF (Molecular Dynamics). CAT activities were normalized for transfection efficiency according to β-galactosidase activities of each extract. β-galactosidase activities of extracts were assayed as described by Rosenthal et al. (Meth. Enzym. 152:704 (1987)).

The results of these studies were as follows. Transfection of the E2-CAT reporter alone or in the presence of the nonfunctional control RB56-H209 mutant yielded relatively high CAT activity. Cotransfection of wild-type RB56 or the variant RB56-5s resulted in a 10 to 12 fold repression of CAT activity, indicating that RB56 or RB56-5s are both capable of efficiently repressing E2F-dependent transcription. E2F194-RB5s and E2F286-RB5s repressed transcription approximately 50 fold. Transcriptional repression required both the RB56 and the E2F components of the fusion proteins, as expression of E2F194 and E2F286 did not mediate transcriptional repression. No repression of SV40-CAT transcription occurred with E2F-RB constructs, thus demonstrating the specificity of the transcriptional repression by E2FRB for the E2 promoter. These results are depicted diagrammatically in Figure 10.

E. Cell cycle arrest

The ability of E2F-RB fusion polypeptides to cause G1 arrest in Saos-2 (RB-/- cells) (ATCC # HTB-85) and C33A cells was investigated. Previous studies have shown that RB-mediated E2 promoter repression and G1 arrest are linked in Saos-2 cells but dissociated in C33A (RBmut) cells (Xu, et al. PNAS **92:1357-1361** (1992)). Cells were washed in PBS and were fixed in 1 mL -20°C 70% ethanol for 30 minutes. Cells were collected by centrifugation and resuspended in 0.5 mL 2% serum containing 10 μg/ml RNase A and incubated for 30 minutes at

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37°C 0.5 mL of PBS containing propidium iodide (100 μg/ml) was added to each sample, mixed and cells were filtered through a FACS tube capstrainer. FACS analysis was performed on a FACS-Scan (Becton-Dickenson) using doublet discrimination. 5,000-10,000 CD20+ events were analyzed. Percent of cells in G_0/G_1, S, and G_2/M was determined using Modfit modeling software.

The results of this experiment were as follows. Both full length RB110 and the truncated version RB56, but not the control mutant RB-H209, caused G_1 arrest in Saos-2 cells (Table 1). Similarly, the RB56-5s, E2F-194-RB56-5s and E2F286-RB56-5s all were capable of arresting cells in G_0/G_1. Transfection of the DNA binding domain, E2F194, did not block S-phase entry in Saos-2 as previously described for rodent cells (Dobrowolski, et al. Oncogene **9:2605-2612** (1994)). In contrast, RB110, RB56, and E2F-RB fusion proteins were not capable of arresting C33A cell lines indicating that the transcriptional repression observed in these cells does not translate into G_1 arrest.

The ability of the E2F-RB fusion proteins to arrest 5637 cells was also investigated (Table 2). RB56 and RB56-5s both efficiently arrested cells in G_0/G_1 (approximately 90% of cells in G_0-G_1), whereas E2F194-RB56-5s and E2F286-RB56-5s are slightly less efficient (about 80% of cells in G_0/G_1) at promoting G_0/G_1 arrest. Without being limited to any one theory, the less efficient arrest of both Saos-2 and 5637 cells by the E2F-RB fusion proteins appears due to the lower levels of steady-state protein produced in these cells (Figure 11, panels b and c).

Table 1: Cell Cycle Regulation by RB and E2F-RB fusion proteins in RBneg cells

	% Cells		
	$CD20^+$ G_0/G_1	G_2/M	S-phase
H209	52.1	27.1	20.8
p56RB	78.8	14.2	7.0
p110RB	70.9	14.3	14.8

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p56RB-5s	84.8	13.2	2.0
p56RB-p5	81.3	11.5	7.3
E2F-194-5s	77.8	14.9	7.3
E2F-286-5s	72.2	15.0	12.8
E2F-194	49.9	28.0	22.1

Table 2: Growth Suppression of 5637 Bladder Cells by RB and E2F-RB fusion proteins

5637/CD20+	% Cells		
	G_0/G_1	S	G,M
CD20	59.7	16.9	20.6
RB56-C706F	57.4	16.3	24.3
RB56WT	90.7	4.12	4.88
RB56-5s	89.91	3.51	6.1
E2F1 94-5s	80.1	1.31	0
E2F-286-5s	79.21	8.1	0

F. Activity of Fusion Proteins in Functional RB Background

The activity of the E2F-RB fusion proteins in a cellular background containing functional RB was then determined. NIH-3T3 cells were transfected with RB56 or E2F-RB56 fusions and stained with anti-RB monoclonal antibody 3C8 (Wen et al. J. Immuno. Meth. **169:231-240** (1994)). FACS analysis was performed of the RB expressing cells. The results are shown in Figure 12. The non-gated population (g) shows the characteristic cell cycle distribution for NIH-3T3 cells (60% GO, 28% S, 10% G2/M). In contrast, in cells transfected with RB56 (a,b) or E2F-RB fusion proteins (c-f), greater than 90% of the RB-expressing cells were arrested in G_0/G_1. These data demonstrate that the ability of RB and E2F-RB56 fusions to arrest cells in G_0/G_1 is not limited to RB negative tumor cells. The relative levels of protein expressed in transfected NIH-3T3 cells was also investigated. RB110 was not expressed efficiently in these cells.

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Thus, these data demonstrate that E2F-RB fusion proteins are more efficient transcriptional repressors than either pRB or RB56 alone, and that RB can repress transcription by remaining bound to E2F rather than directly blocking the transactivation domain of E2F. These data support the use of E2F-RB fusions as RB agonists in both RB+ cells and in RB negative or RB mutant cells.

Example II.

Tissue-Specific Expression of E2F-RB Fusions

A. Construction of Recombinant Adenovirus:

In this experiment, recombinant adenoviruses comprising an RB polypeptide under the control of a CMV or smooth muscle alpha actin promoter were generated.

The smooth muscle α-actin promoter (bases -670 through +5, Reddy et al. "Structure of the Human Smooth Muscle α-Actin Gene." J. Biol. Chem. **265:1683-1687** (1990), Nakano, et al. "Transcriptional Regulatory Elements In The 5' Upstream and First Intron Regions of The Human Smooth Muscle (aortic type) α-Actin-Encoding Gene." Gene **99:285-289** (1991) was isolated by PCR from a genomic library with 5' Xho I and Avr II and 3' Xba I, Cla I and Hind III restriction sites added for cloning purposes. The fragment was subcloned as an Xho 1, Hind III fragment into a plasmid for sequencing to verify base composition. A fusion construct 286-56 containing the DNA and heterodimerization domain of E2F-1 (bases 95-286) linked to p56 (amino acids 379-928 of full length RB) was subcloned as an Xba I, Cla I fragment directly downstream of the smooth muscle α-actin promoter, and this expression cassette was digested out and cloned into the plasmid pAd/ITR/IX- as an Xba I to AvrII, and Cla I fragment to create the plasmid pASN286-56. This plasmid consisted of the adenovirus type 5 inverted terminal repeat (ITR), packaging signals and Ela enhancer, followed by the human smooth muscle α-actin promoter and 286-56 cassette, and then Ad 2 sequence 4021-10462 (which contains the E1b/protein IX poly A signal) in a pBR322 background. Recombinant adenovirus was produced by standard procedures.

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The plasmid pASN286-56 was linearized with Ngo MI and co-transfected into 293 cells with the large fragment of Cla I digested rAd34 which has deletions in both the E3 and E4 regions of adenovirus type 5. Ad34 was a serotype 5 derivative with a 1.9 KB deletion in early region 3 resulting from deletion of the Xba I restriction fragment extending from Ad5 coordinates 28593 to 30470 and a 1.4 KB deletion of early region 4 resulting from a Taq 1 fragment of E4 (coordinates 33055-35573) being replaced with a cDNA containing E4 ORF 6 and 6/7.

Recombinant adenovirus produced by homologous recombination was isolated and identified by restriction digest analysis and further purified by limiting dilution. Additional control recombinant adenoviruses are described elsewhere and include the control virus ACN (CMV promoter, Wills, et al. "Gene Therapy For Hepatocellular Carcinoma: Chemosensitivity Conferred By Adenovirus-Mediated Transfer of The HSV-1 Thymidine Kinase Gene." Cancer Gene Therapy **2:191-197** (1995)), and ACN56 (RB expressed under control of a CMV promoter).

ACN56 was prepared as follows. A plasmid containing p56 cDNA was constructed by replacing the p53 cDNA from the plasmid ACNP53 (Wills et al. Human Gene Therapy 5:1079-1088 (1994)) with a 1.7 KB Xba I- BamHI fragment isolated from plasmid pET 9a-Rb56 (Antelman et al. Oncogene 10:697-704 (1995)) which contains p56 cDNA. The resulting plasmid contained amino acids 381-928 of p56, the Ad5 inverted terminal repeat, viral packaging signals and E1a enhancer, followed by the human cytomegalovirus immediate early promoter (CMV) and Ad 2 tripartite leader cDNA to drive p56 expression. The p56 cDNA was followed by Ad 2 sequence 4021-10462 in a pBR322 background. This plasmid was linearized with EcoRI and cotransfected with the large fragment of bsp 106 digested DL327 (E3 deleted; Thimmappaaya et al. Cell 31:543-551 (1982)) or h5ile4 (E4 deleted; Hemstrom et al. J. Virol. 62:3258-3264 (1988)). Recombinant viruses were further purified by limiting dilution.

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B. Cellular Proliferation

In this experiment, cell lines were infected in culture with recombinant adenovirus RB constructs to ascertain the relative expression of the RB polypeptide and the effect on cell proliferation.

For H358 (ATCC # Crl 5807) and MDA-MB468 (ATCC # HTB 132, breast adenocarcinoma) cells, 5,000 cell/well were plated in normal growth media in a 96 well microtiter plate (Costar) and allowed to incubate overnight at 37°C, 7% CO_2. Viruses were serially diluted in growth media and used to infect cells at the indicated doses for 48 hours. At this point, ^{3}H-thymidine was added (Amersham, 0.5 μCi/well) and the cells were incubated at 37°C for another 3 hours prior to harvest. Both A7r5 (ATCC CRL1444, rat smooth muscle) and A10 (ATCC CRL 1476, rat smooth muscle) cells were seeded at 3,000 cells/well in either DME + 0.5% FCS or DME + 20% FCS respectively. Virus was serially diluted in the seeding media and used to infect the cells at the doses indicated in the Figures. The infection and labelling procedure were the same for A10 cells as with the H358 and MDA-MB468 cells except that 2 μCi/well of label was used. The A7r5 cells were not infected with virus until 48 hours after seeding. Forty eight hours after infection, the serum concentration was raised to 10% FCS and 2 μCi/well of ^{3}H-thymidine was added and incubation continued for an additional 3 hours prior to harvest. All cells were harvested by aspirating media from the wells, trypsinization of the cells, and harvesting using a 96 well GF/C filter with a Packard Top count cell harvester. Results are plotted as the mean percentage (+/- SD) of media treated control proliferation versus dose of virus in Figures 13 and 14.

Thus, Figure 13 depicts a comparison of the effects of adenovirus p56 constructs on muscle cells A10 and A7R5 cells. The CMV-driven p56 (ACN 56) virus inhibited A10 growth to approximately the same extent as the actin promoter-driven E2F-fusion constructs (ASN586-56 #25,26). In Figure 14, the effects of adenovirus constructs on inhibition of a breast cancer cell line, MDA Mβ468 and a non- small cell lung carcinoma cell line, H358, are depicted. In these

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experiments, actin promoter-driven E2F-p56 was ineffective, while the CMV promoter-driven p56 was effective in inhibiting growth of non-smooth muscle cells.

To determine whether the non-smooth muscle cells were more infectable with adenovirus than the smooth muscle cell lines used, the four cells lines, H358, MB468, A7R5, and A10 were infected at an MOI of 5 with an adenovirus expressing β-galactosidase (ACβGL; Wills, et al. Human Gene Therapy **5:1079-1088** (1994)) and degree of β-gal staining was examined. As shown in Figure 15 (top), the non-smooth muscle cell lines were significantly more infectable than the smooth muscle cell lines. In a further test, cells were infected at higher multiplicities of infection (50, 100, 250, 500) with ACN56 and the amount of p56 present in the infected cells detected by autoradiography. As can be seen in Figure 15 (bottom), the non-muscle cell lines had significantly more p56 present, since as a result of their greater infectivity, infected cells have a greater viral load and thus more copies of the p56 template driven by the non-tissue specific CMV promoter.

In a further experiment, the specificity of the actin smooth muscle promoter for smooth muscle tissue was ascertained. In this experiment, β-gal expression levels in cells infected with β-gal constructs driven with different promoters were measured. As can be seen in Figure 19, despite the lower infectivity of the smooth muscle cells, expression was only evident in these cells using the smooth muscle alpha actin promoter.

Figure 21 depicts a comparison of the effects of a CMV driven p56 recombinant adenovirus (ACN56E4) vs a human smooth muscle alpha-actin promoter driven E2F-p56 fusion construct (ASN286-56) vs control adenoviral construct containing either the CMV or smooth muscle alpha-actin promoters without a downstream transgene (ACNE3 or ASBE3-2 isolates shown, respectively). Assays were 3H-thymidine uptake either in a smooth muscle cell line (A7R5) or a non-muscle cell line (MDA-MB468, breast carcinoma). Results demonstrated muscle tissue specificity using the smooth muscle

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alpha-actin promoter and specific inhibition of both the p56 and E2F-p56 transgenes relative to their respective controls.

C. Inhibition of Restenosis

The model of balloon injury was based on that described by Clowes, et al. (Clowes, Lab. Invest. 49:327-333 (1983)). Male Sprague-Dawley rats weighing 400-500g were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg. Abbot Laboratories, North Chicago, Illinois). The bifurcation of the left common carotid artery was exposed through a midline incision and the left common, internal, and external carotid arteries were temporarily ligated. A 2F embolectomy catheter (Baxter Edwards Healthcare Corp., Irvine, CA) was introduced into the external carotid and advanced to the distal ligation of the common carotid. The balloon was inflated with saline and drawn towards the arteriotomy site 3 times to produce a distending, deendothelializing injury. the catheter was then withdrawn. Adenovirus (1 x 10^9 pfu of Ad-RB (ACNRb) or Ad-p56 (ACN56) in a volume of 10μl diluted to 100μl with 15% (wt/vol) Poloxamer 407 (BASF, Parsippany, N.J.) or Ad-β-Gal (1 x 10^9 pfu, diluted as above) was injected via a canula, inserted just proximal to the carotid bifurcation into a temporarily isolated segment of the artery. The adenovirus solution was incubated for 20 minutes after which the viral infusion was withdrawn and the cannula removed. The proximal external carotid artery was then ligated and blood flow was restored to the common carotid artery by release of the ligatures. The experimental protocol was approved by the Institutional Animal Care and Use Committee and complied with the "Guide for the Care and Use of Laboratory Animals." (NIH Publication No. 86-23, revised 1985).

Rats were sacrificed at 14 days following treatment with an intraperitoneal injection of pentobarbital (100 mg/kg.). The initially balloon injured segment of the left common carotid artery, from the proximal edge of the omohyoid muscle to the carotid bifurcation, was perfused with saline and dissected free of the surrounding tissue. The tissue was

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fixed in 100% methanol until imbedded in paraffin. Several 4-μm sections were cut from each tissue specimen. One section from each specimen was stained with hematoxylin and eosin and another with Richardson's combination elastic-trichrome stain conventional light microscopic analysis.

Histological images of cross sections of hematoxylin and eosin or elastic-trichrome stained arterial sections were projected onto a digitizing board (Summagraphics) and the intimal, medial and luminal areas were measured by quantitative morphometric analysis using a computerized sketching program (MACMEASURE, version 1.9, National Institute of Mental Health).

Results were expressed as the mean ± S.E.M. Differences between groups were analyzed using an unpaired two-tailed Student's *t* test. Statistical significance was assumed when the probability of a null effect was <0.05.

Results are shown in Figures 17 and 18. In Figure 17, the relative inhibition of neointima formation is depicted graphically, demonstrating the ability of p56 and RB to inhibit neointima formation. Figure 18 provides photographic evidence of the dramatic reduction of neointima in the presence of p56.

Adenovirus-treated carotid arteries were harvested from rats at 2 days following balloon injury and infections. Tissue was fixed in phosphate-buffered formalin until embedded in paraffin. Tissue was cut into 4μm cross-sections and dewaxed through xylene and graded alcohols. Endogenous peroxidase was quenched with 1% hydrogen peroxide for 30 minutes. Antigen retrieval was performed in 10mM sodium citrate buffer, pH 6.0 at 95°C for 10 minutes. A monoclonal anti-RB antibody (AB-5, Oncogene Sciences, Uniondale, New York) was applied 10μg/ml in PBS in a humid chamber at 4°C for 24 hours. Secondary antibody was applied from the Unitect Mouse Immunohistochemistry Kit (Oncogene Sciences, Uniondale, New York) according to the manufacturer's instructions. The antibody complexes were visualized using 3,3'-diaminobenzidene (DAB, Vector Laboratories, Burlingame, CA). Slides were thin

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counterstained with hematoxylin and mounted. The results are depicted in Figure 20.

All references cited herein are hereby incorporated by reference in their entirety for all purposes.

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WHAT IS CLAIMED IS:

1. A polypeptide comprising a fusion of a transcription factor, the transcription factor comprising a DNA binding domain, and a retinoblastoma (RB) polypeptide, the RB polypeptide comprising a growth suppression domain.

2. A nucleic acid encoding the fusion polypeptide of claim 1.

3. The nucleic acid of claim 2, wherein the nucleic acid in inserted in an adenovirus vector.

4. The polypeptide of claim 1, wherein the transcription factor is E2F.

5. The polypeptide of claim 4, wherein the cyclin A binding domain of the E2F is deleted or nonfunctional.

6. The polypeptide of claim 1, wherein the retinoblastoma polypeptide is RB56.

7. The polypeptide of claim 1, wherein the retinoblastoma polypeptide is wild type RB.

8. The polypeptide of claim 1, wherein the retinoblastoma polypeptide comprises from about amino acid residue 379 to about amino acid residue 928 of pRB.

9. The polypeptide of claim 1, wherein the retinoblastoma polypeptide comprises at least one substitution of amino acid residues selected from the group consisting of 2, 608, 612, 788, 807, and 811 of pRB.

10. The polypeptide of claim 5, wherein the E2F comprises about amino acid residues 95 to about 286.

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11. The polypeptide of claim 4, wherein the E2F comprises about amino acid residues 95 to about 194.

12. The polypeptide of claim 1, wherein the fusion comprises EF2 amino acid residues from about 95 to about 194 operatively linked to RB amino acid residues from about 379 to about 928.

13. An expression vector comprising DNA encoding a polypeptide, the polypeptide comprising a fusion of a transcription factor, the transcription factor comprising a DNA binding domain, and a retinoblastoma (RB) polypeptide, the RB polypeptide comprising a growth suppression domain.

14. The vector of claim 13, comprising a tissue-specific promoter operatively linked to DNA encoding the fusion.

15. The vector of claim 14, wherein the tissue specific promoter is a smooth muscle actin promoter.

16. A method for treatment of a hyperproliferative disorder in a patient comprising administering to a patient a therapeutically effective dose of a fusion polypeptide comprising a fusion of a transcription factor, the transcription factor comprising a DNA binding domain, and a retinoblastoma (RB) polypeptide, the RB polypeptide comprising a growth suppression domain.

17. The method of claim 16, wherein the fusion protein is encoded by a nucleic acid delivered to the patient.

18. The method of claim 16, wherein the transcription factor is E2F.

19. The method of claim 18, wherein the cyclin A binding domain of the E2F is deleted or nonfunctional.

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20. The method of claim 16, wherein the RB is RB56.

21. The method of claim 16, wherein the RB is wild type RB56.

22. The method of claim 16, wherein the RB comprises from about amino acid residue 379 to about amino acid residue 928.

23. The method of claim 16, wherein the RB comprises at least one substitution of amino acid residues selected from the group consisting of 2, 608, 612, 788, 807, and 811.

24. The method of claim 18, wherein the E2F comprises about amino acid residues 95 to about 286.

25. The method of claim 18, wherein the E2F comprises about amino acid residues 95 to about 194.

26. The method of claim 16, wherein the fusion comprises EF2 amino acid residues from about 95 to about 194 operatively linked to RB amino acid residues from about 379 to about 928.

27. The method of claim 18, wherein the E2F -RB fusion polypeptide is expressed under the control of a tissue-specific promoter.

28. The method of claim 27, wherein the tissue specific promoter is a smooth muscle actin promoter.

29. The method of claim 16, wherein the hyperproliferative disorder is cancer.

30. The method of claim 29, wherein the cancer is bladder cancer.

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31. The method of claim 29, wherein the hyperproliferative disorder is restenosis.

32. The method of claim 31, wherein the E2F-RB fusion polypeptide is administered after angioplasty.

33. The method of claim 32, wherein the E2F-RB fusion polypeptide is administered as a coating on an angioplasty device.

34. The method of claim 17, wherein the nucleic acid is administered after angioplasty.

35. The method of claim 17, wherein the nucleic acid is administered as a coating on an angioplasty device.

36. The method of claim 17, wherein the nucleic acid is inserted in an adenovirus vector.

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```
        10          20          30          40          50          60
MALAGAPAGG PCAPALEALL GAGALRLLDS SQIVIISAAQ DASAPPAPTG PAAPAAGPCD

        70          80          90         100         110         120
PDLLLFATPQ APRPTPSAPR PALGRPPVKR RLDLETDHQY LAESSGPARG RGRHPGKGVK

       130         140         150         160         170         180
SPGEKSRYET SLNLTTKRFL ELLSHSADGV VDLNWAAEVL KVQKRRIYDI TNVLEGIQLI

       190         200         210         220         230         240
AKKSKNHIQW LGSHTTVGVG GRLEGLTQDL RQLQESEQQL DHLMNICTTQ LRLLSEDTDS

       250         260         270         280         290         300
QRLAYVTCQD LRSIADPAEQ MVMVIKAPPE TQLQAVDSSE NFQISLKSKQ GPIDVFLCPE

       310         320         330         340         350         360
ETVGGISPGK TPSQEVTSEE ENRATDSATI VSPPPSSPPS SLTTDPSQSL LSLEQEPLLS

       370         380         390         400         410         420
RMGSLRAPVD EDRLSPLVAA DSLLEHVRED FSGLLPEEFI SLSPPHEALD YHFGLEEGEG

       430         440         450         460         470         480
IRDLFDCDFG DLTPLDF*.. .......... .......... .......... ..........
```

FIG. 1A

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```
        10          20          30          40          50          60
GGAATTCCGT GGCCGGGACT TTGCAGGCAG CGGCGGCCGG GGGCGGAGCG GGATCGAGCC

        70          80          90         100         110         120
CTCGCCGAGG CCTGCCGCCA TGGGCCCGCG CCGCCGCCGC CGCCTGTCAC CCGGGCCGCG

       130         140         150         160         170         180
CGGGCCGTGA GCGTCATGGC CTTGGCCGGG GCCCCTGCGG GCGGCCCATG CGCGCCGGCG

       190         200         210         220         230         240
CTGGAGGCCC TGCTCGGGGC CGGCGCGCTG CGGCTGCTCG ACTCCTCGCA GATCGTCATC

       250         260         270         280         290         300
ATCTCCGCCG CGCAGGACGC CAGCGCCCCG CCGGCTCCCA CCGGCCCCGC GGCGCCCGCC

       310         320         330         340         350         360
GCCGGCCCCT GCGACCCTGA CCTGCTGCTC TTCGCCACAC CGCAGGCGCC CCGGCCCACA

       370         380         390         400         410         420
CCCAGTGCGC CGCGGCCCGC GCTCGGCCGC CCGCCGGTGA AGCGGAGGCT GGACCTGGAA

       430         440         450         460         470         480
ACTGACCATC AGTACCTGGC CGAGAGCAGT GGGCCAGCTC GGGGCAGAGG CCGCCATCCA

       490         500         510         520         530         540
GGAAAAGGTG TGAAATCCCC GGGGGAGAAG TCACGCTATG AGACCTCACT GAATCTGACC

       550         560         570         580         590         600
ACCAAGCGCT TCCTGGAGCT GCTGAGCCAC TCGGCTGACG GTGTCGTCGA CCTGAACTGG

       610         620         630         640         650         660
GCTGCCGAGG TGCTGAAGGT GCAGAAGCGG CGCATCTATG ACATCACCAA CGTCCTTGAG

       670         680         690         700         710         720
GGCATCCAGC TCATTGCCAA GAAGTCCAAG AACCACATCC AGTGGCTGGG CAGCCACACC

       730         740         750         760         770         780
ACAGTGGGCG TCGGCGGACG GCTTGAGGGG TTGACCCAGG ACCTCCGACA GCTGCAGGAG

       790         800         810         820         830         840
AGCGAGCAGC AGCTGGACCA CCTGATGAAT ATCTGTACTA CGCAGCTGCG CCTGCTCTCC

       850         860         870         880         890         900
GAGGACACTG ACAGCCAGCG CCTGGCCTAC GTGACGTGTC AGGACCTTCG TAGCATTGCA

       910         920         930         940         950         960
GACCCTGCAG AGCAGATGGT TATGGTGATC AAAGCCCCTC CTGAGACCCA GCTCCAAGCC

       970         980         990        1000        1010        1020
GTGGACTCTT CGGAGAACTT TCAGATCTCC CTTAAGAGCA AACAAGGCCC GATCGATGTT

      1030        1040        1050        1060        1070        1080
TTCCTGTGCC CTGAGGAGAC CGTAGGTGGG ATCAGCCCTG GGAAGACCCC ATCCCAGGAG

      1090        1100        1110        1120        1130        1140
GTCACTTCTG AGGAGGAGAA CAGGGCCACT GACTCTGCCA CCATAGTGTC ACCACCACCA

      1150        1160        1170        1180        1190        1200
TCATCTCCCC CCTCATCCCT CACCACAGAT CCCAGCCAGT CTCTACTCAG CCTGGAGCAA

      1210        1220        1230        1240        1250        1260
GAACCGCTGT TGTCCCGGAT GGGCAGCCTG CGGGCTCCCG TGGACGAGGA CCGCCTGTCC
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FIG. 1B

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      1330        1340        1350        1360        1370        1380
CCTGAGGAGT TCATCAGCCT TTCCCCACCC CACGAGGCCC TCGACTACCA CTTCGGCCTC

      1390        1400        1410        1420        1430        1440
GAGGAGGGCG AGGGCATCAG AGACCTCTTC GACTGTGACT TTGGGGACCT CACCCCCCTG

      1450        1460        1470        1480        1490        1500
GATTTCTGAC AGGGCTTGGA GGGACCAGGG TTTCCAGAGT AGCTCACCTT GTCTCTGCAG

      1510        1520        1530        1540        1550        1560
CCCTGGAGCC CCCTGTCCCT GGCCGTCCTC CCAGCCTGTT TGGAAACATT TAATTTATAC

      1570        1580        1590        1600        1610        1620
CCCTCTCCTC TGTCTCCAGA AGCTTCTAGC TCTGGGGTCT GGCTACCGCT AGGAGGCTGA

      1630        1640        1650        1660        1670        1680
GCAAGCCAGG AAGGGAAGGA GTCTGTGTGG TGTGTATGTG CATGCAGCCT ACACCCACAC

      1690        1700        1710        1720        1730        1740
GTGTGTACCG GGGGTGAATG TGTGTGAGCA TGTGTGTGTG CATGTACCGG GGAATGAAGG

      1750        1760        1770        1780        1790        1800
TGAACATACA CCTCTGTGTG TGCACTGCAG ACACGCCCCA GTGTGTCCAC ATGTGTGTGC

      1810        1820        1830        1840        1850        1860
ATGAGTCCAT CTCTGCGCGT GGGGGGGCTC TAACTGCACT TTCGGCCCTT TTGCTCGTGG

      1870        1880        1890        1900        1910        1920
GGTCCCACAA GGCCCAGGGC AGTGCCTGCT CCCAGAATCT GGTGCTCTGA CCAGGCCAGG

      1930        1940        1950        1960        1970        1980
TGGGGAGGCT TTGGCTGGCT GGGCGTGTAG GACGGTGAGA GCACTTCTGT CTTAAAGGTT

      1990        2000        2010        2020        2030        2040
TTTTCTGATT GAAGCTTTAA TGGAGCGTTA TTTATTTATC GAGGCCTCTT TGGTGAGCCT

      2050        2060        2070        2080        2090        2100
GGGGAATCAG CAAAAGGGGA GGAGGGGTGT GGGGTTGATA CCCCAACTCC CTCTACCCTT

      2110        2120        2130        2140        2150        2160
GAGCAAGGGC AGGGGTCCCT GAGCTGTTCT TCTGCCCCAT ACTGAAGGAA CTGAGGCCTG

      2170        2180        2190        2200        2210        2220
GGTGATTTAT TTATTGGGAA AGTGAGGGAG GGAGACAGAC TGACTGACAG CCATGGGTGG

      2230        2240        2250        2260        2270        2280
TCAGATGGTG GGGTGGGCCC TCTCCAGGGG GCCAGTTCAG GGCCCAGCTG CCCCCCAGGA

      2290        2300        2310        2320        2330        2340
TGGATATGAG ATGGGAGAGG TGAGTGGGGG ACCTTCACTG ATGTGGGCAG GAGGGGTGGT

      2350        2360        2370        2380        2390        2400
GAAGGCCTCC CCCAGCCCAG ACCCTGTGGT CCCTCCTGCA GTGTCTGAAG CGCCTGCCTC

      2410        2420        2430        2440        2450        2460
CCCACTGCTC TGCCCCACCC TCCAATCTGC ACTTTGATTT GCTTCCTAAC AGCTCTGTTC

      2470        2480        2490        2500        2520        2520
CCTCCTGCTT TGGTTTTAAT AAATATTTTG ATGACGTTAA AAAAAGGAAT TCGATAT
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FIG. 1B
(CONTINUED)

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2041 gccttccaga cccagaagcc attgaaatct acctctcttt cactgtttta taaaaaagtg
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2161 gaattagaac atatcatctg gacccttttc cagcacaccc tgcagaatga gtatgaactc
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FIG. 2A

ISDOCID: <WO___9821228A1_IA>

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MLCVLDYFIKLSPPMLLKEPYKTAVIPINGSPRTPRRGQNRSARIAKQLENDTRIIEV
LCKEHECNIDEVKNVYFKNFIPFMNSLGLVTSNGLPEVENLSKRYEEIYLKNKDLDAR
LFLDHDKTLQTDSIDSFETQRTPRKSNLDEEVNVIPPHTPVRTVMNTIQQLMMILNSA
SDQPSENLISYFNNCTVNPKESILKRVKDIGYIFKEKFAKAVGQGCVEIGSQRYKLGV
RLYYRVMESMLKSEEERLSIQNFSKLLNDNIFHMSLLACALEVVMATYSRSTSQNLDS
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IPGGNIYISPLKSPYKISEGLPTPTKMTPRSRILVSIGESFGTSEKFQKINQMVCNSD
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FIG. 2B

DOCID: <WO___9821228A1_IA>

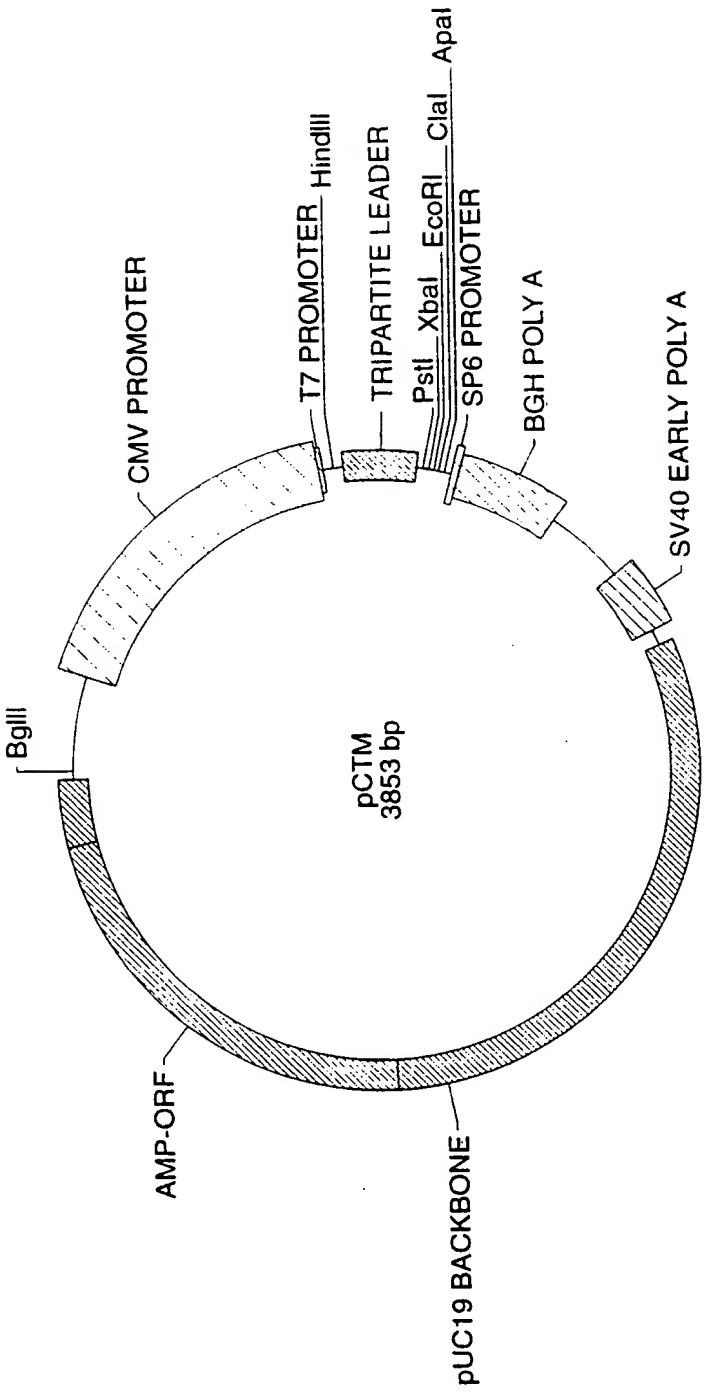

FIG. 3

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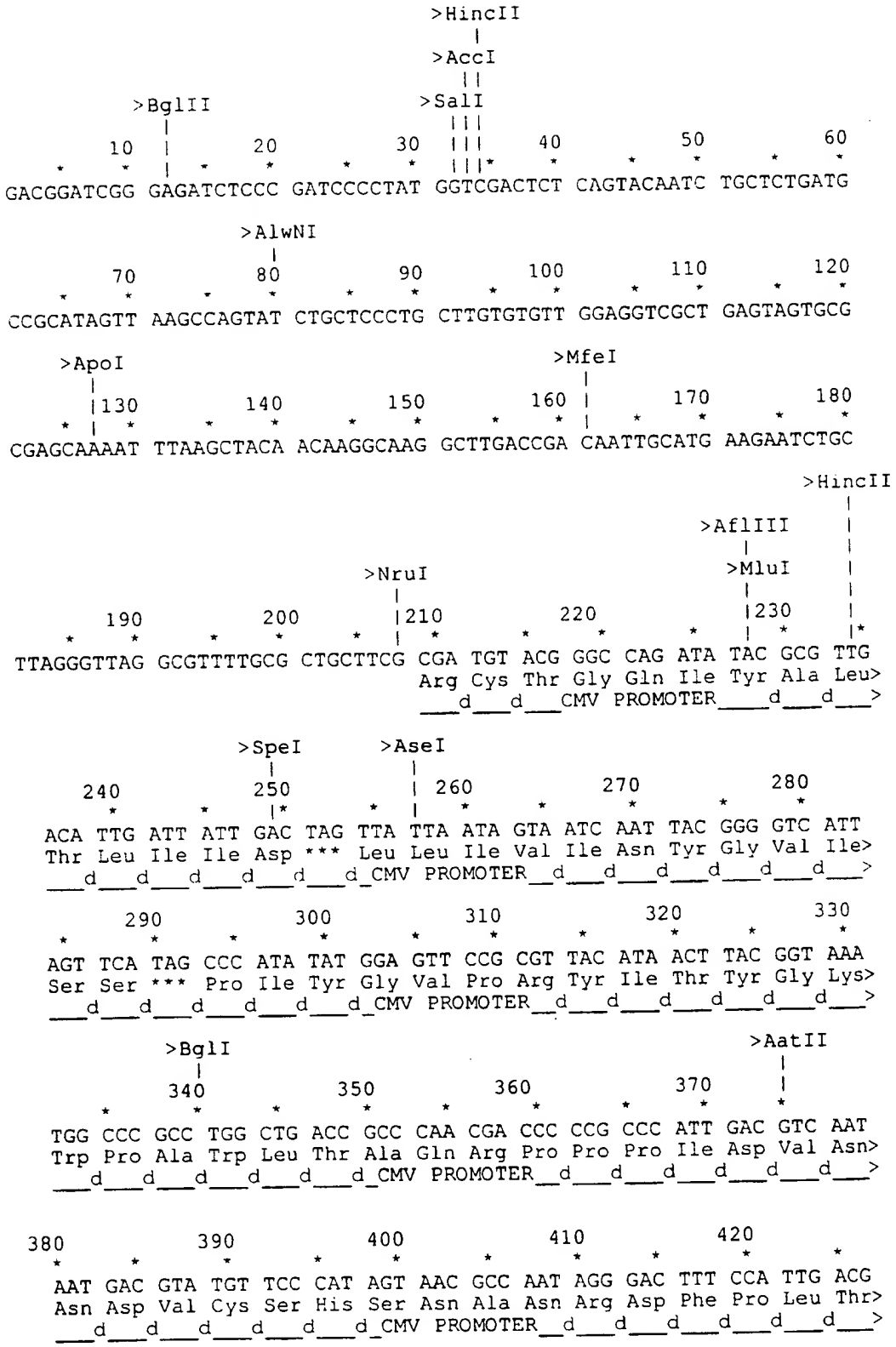

FIG. 4

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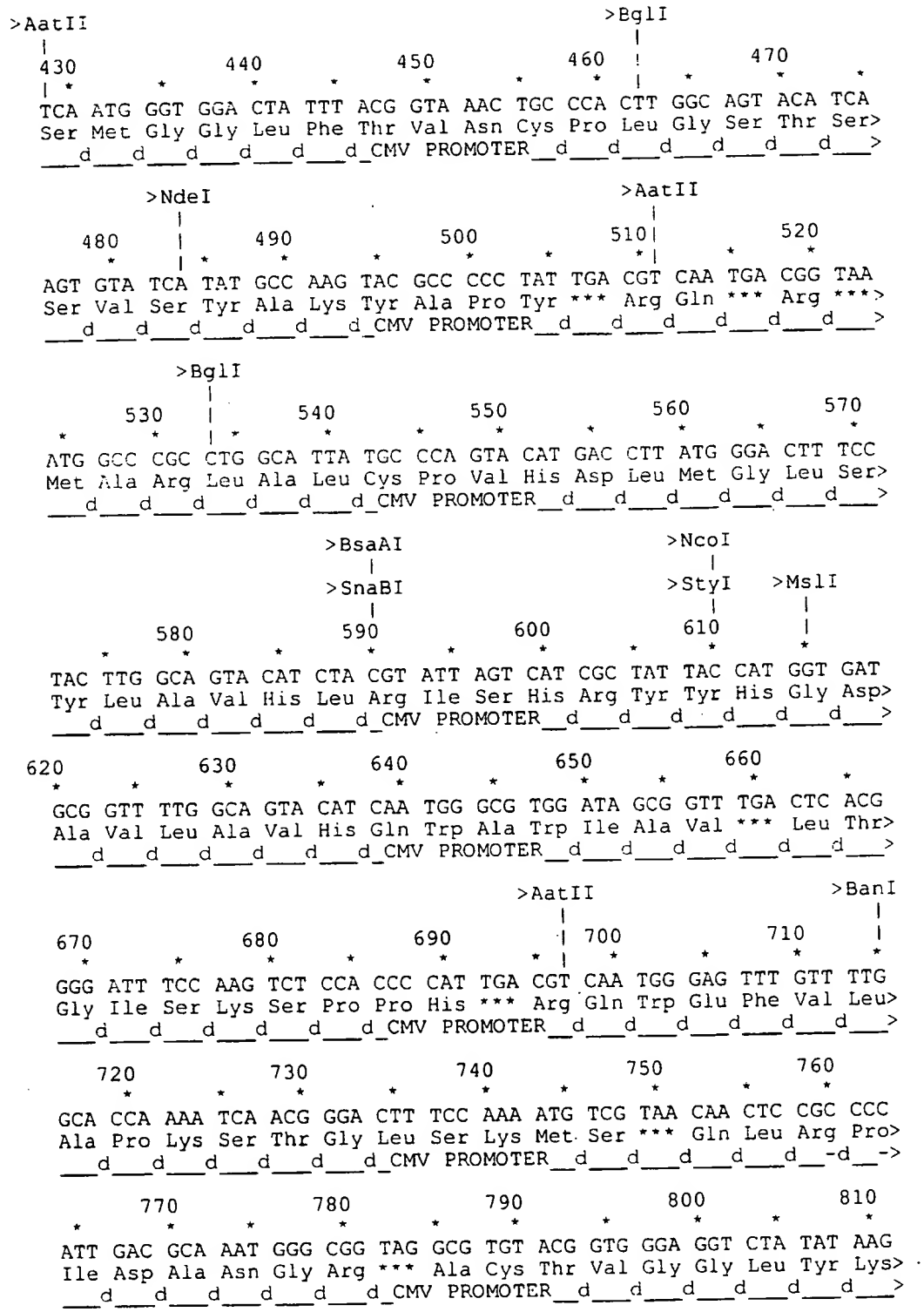

FIG. 4
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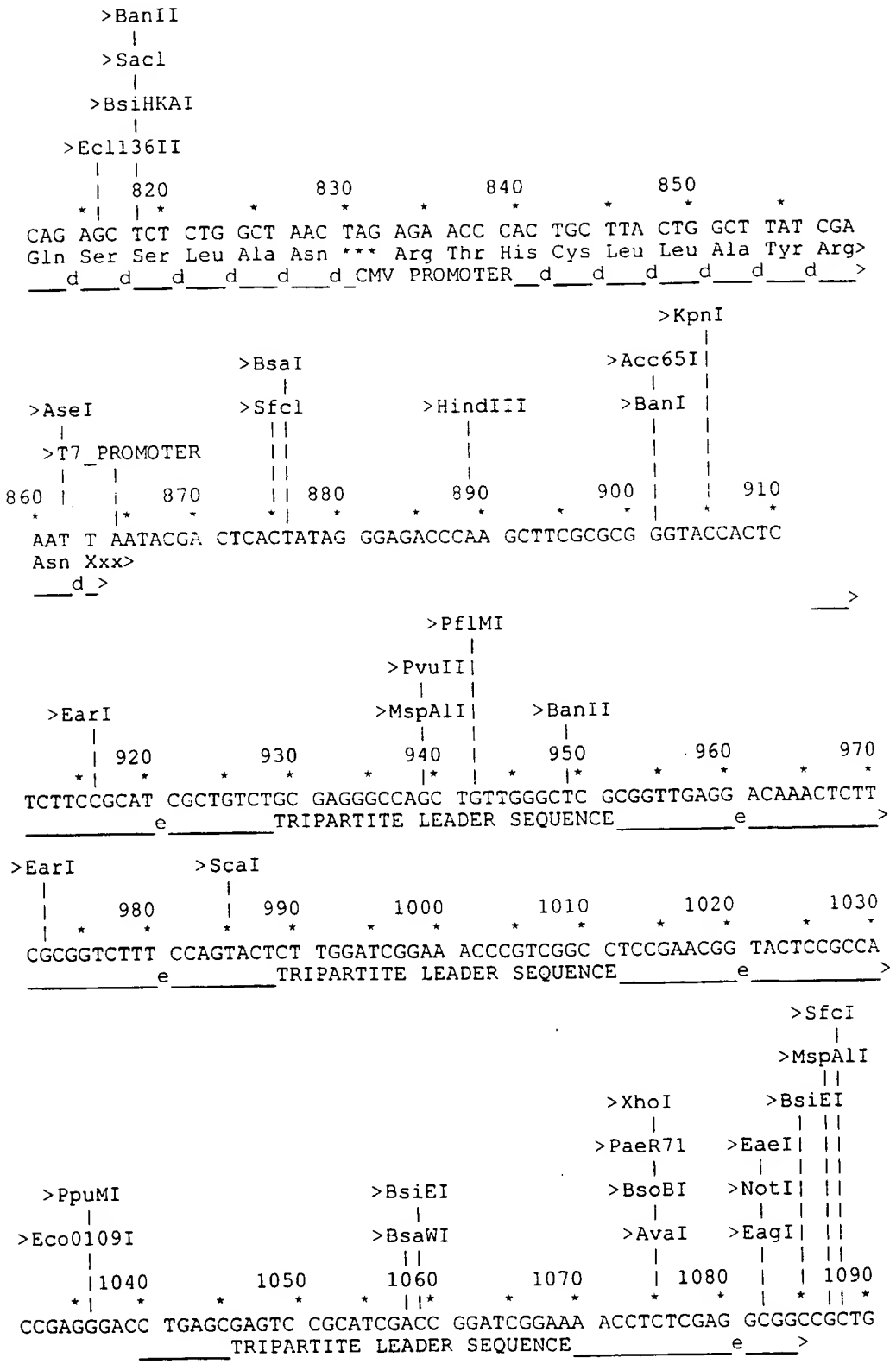

FIG. 4
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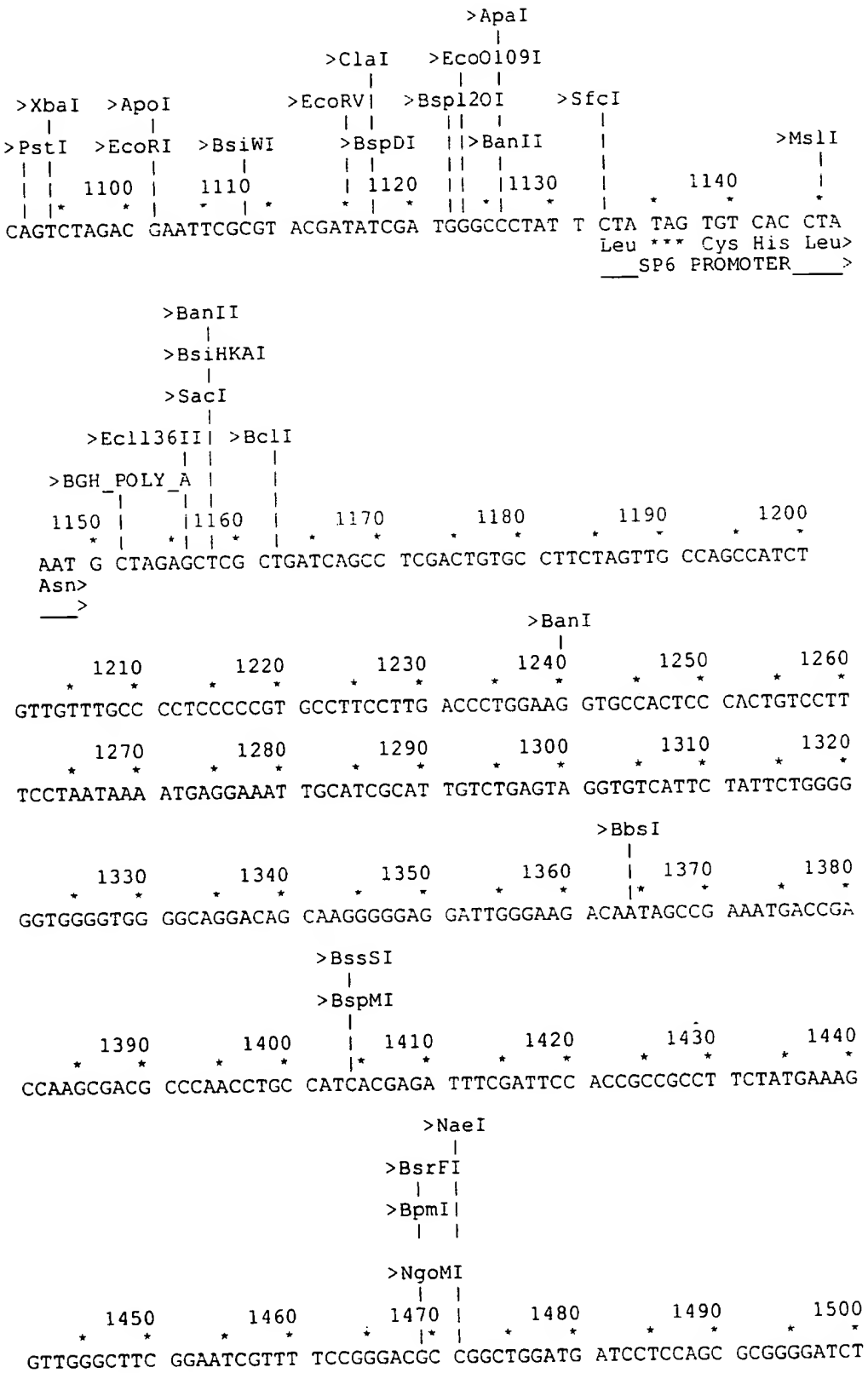

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(CONTINUED)

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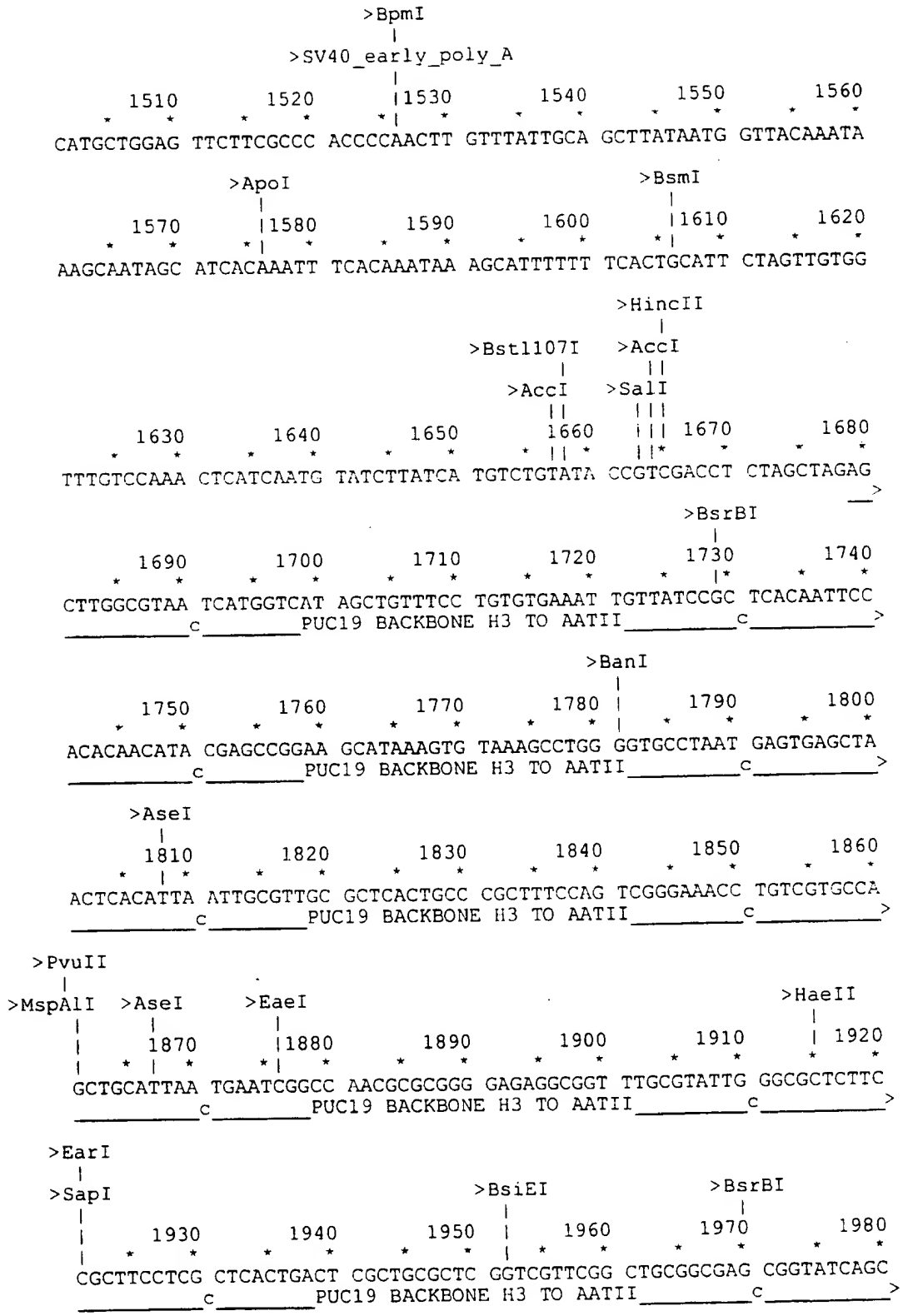

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(CONTINUED)

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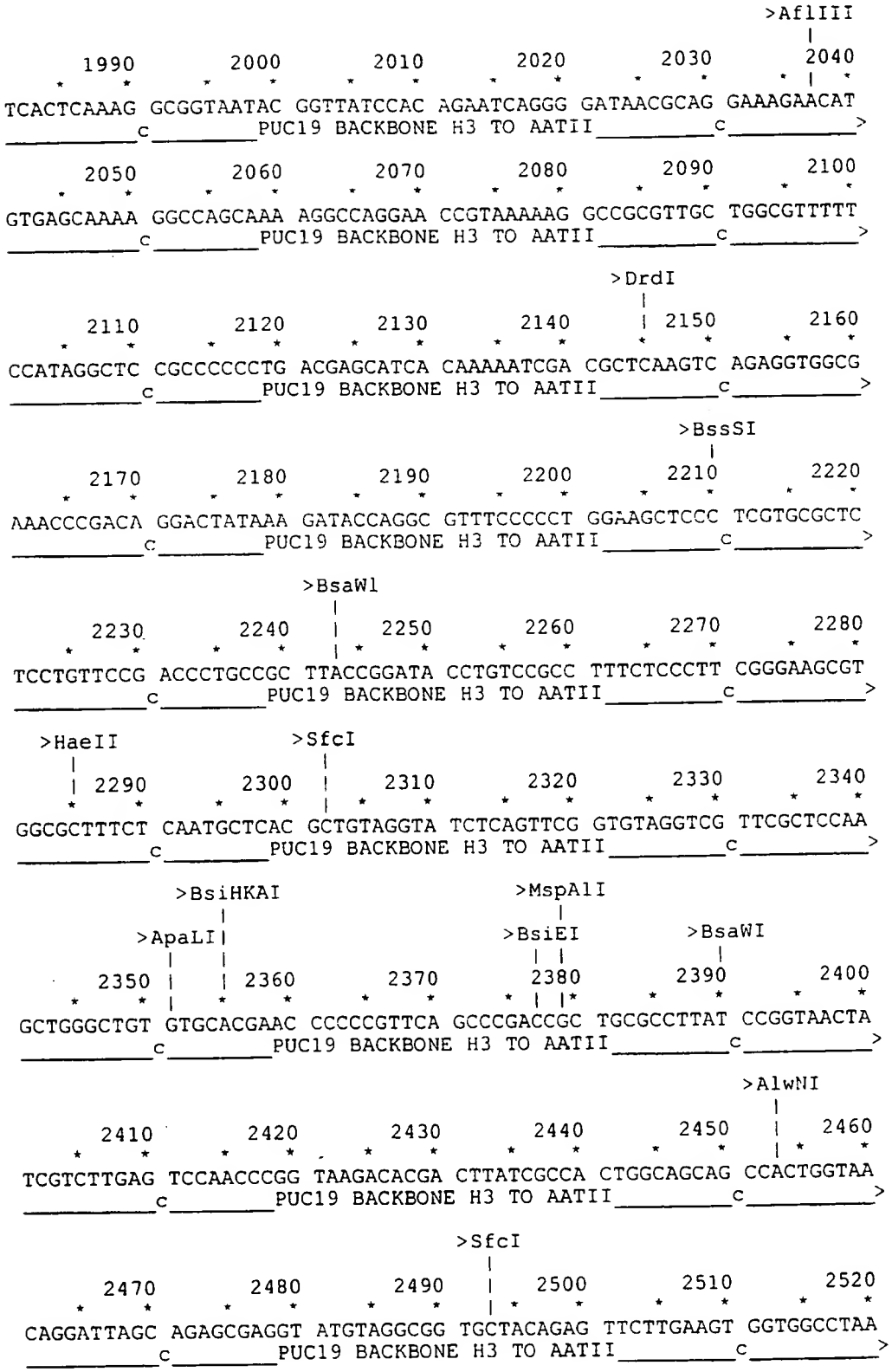

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(CONTINUED)

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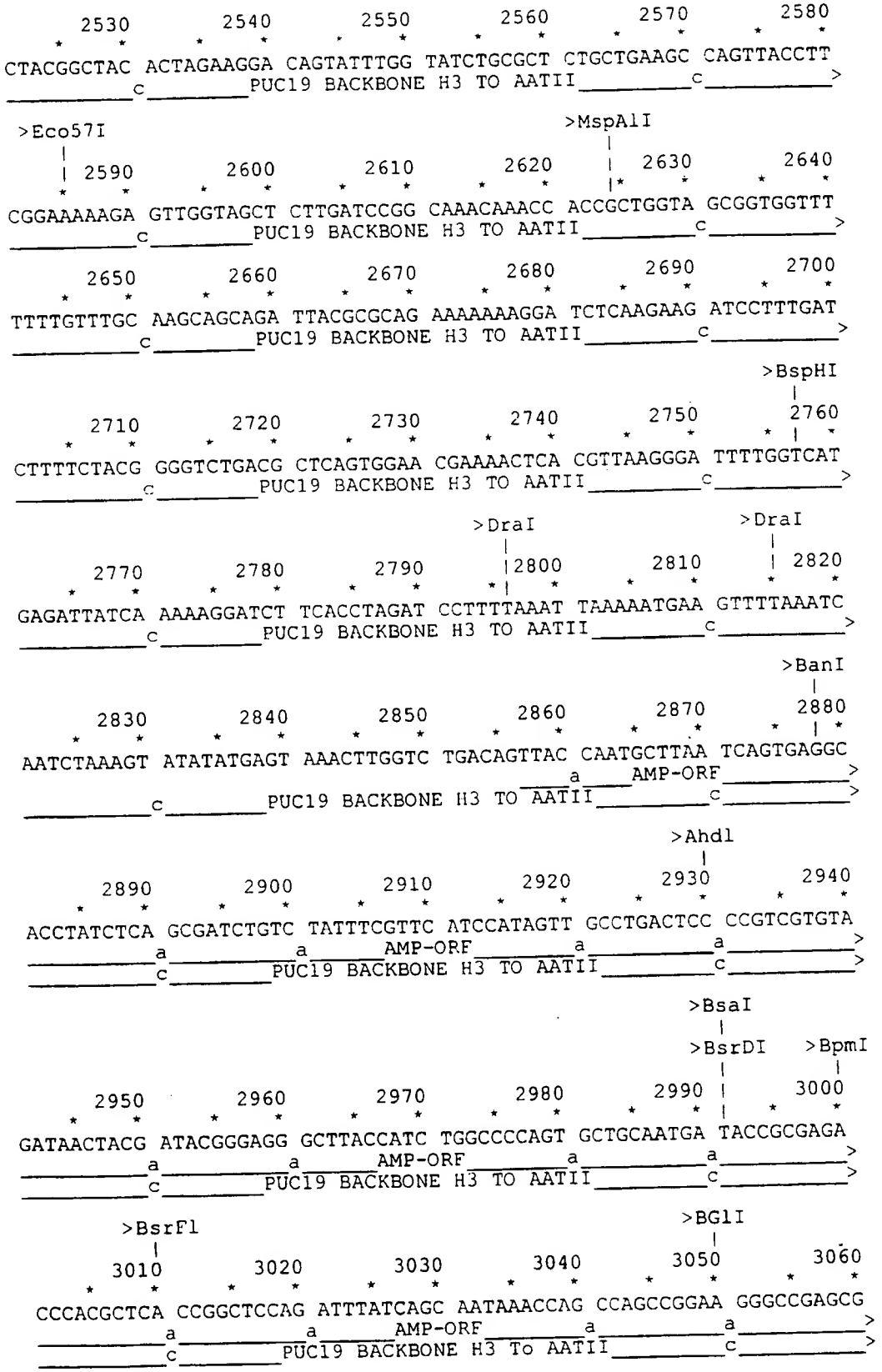

FIG. 4
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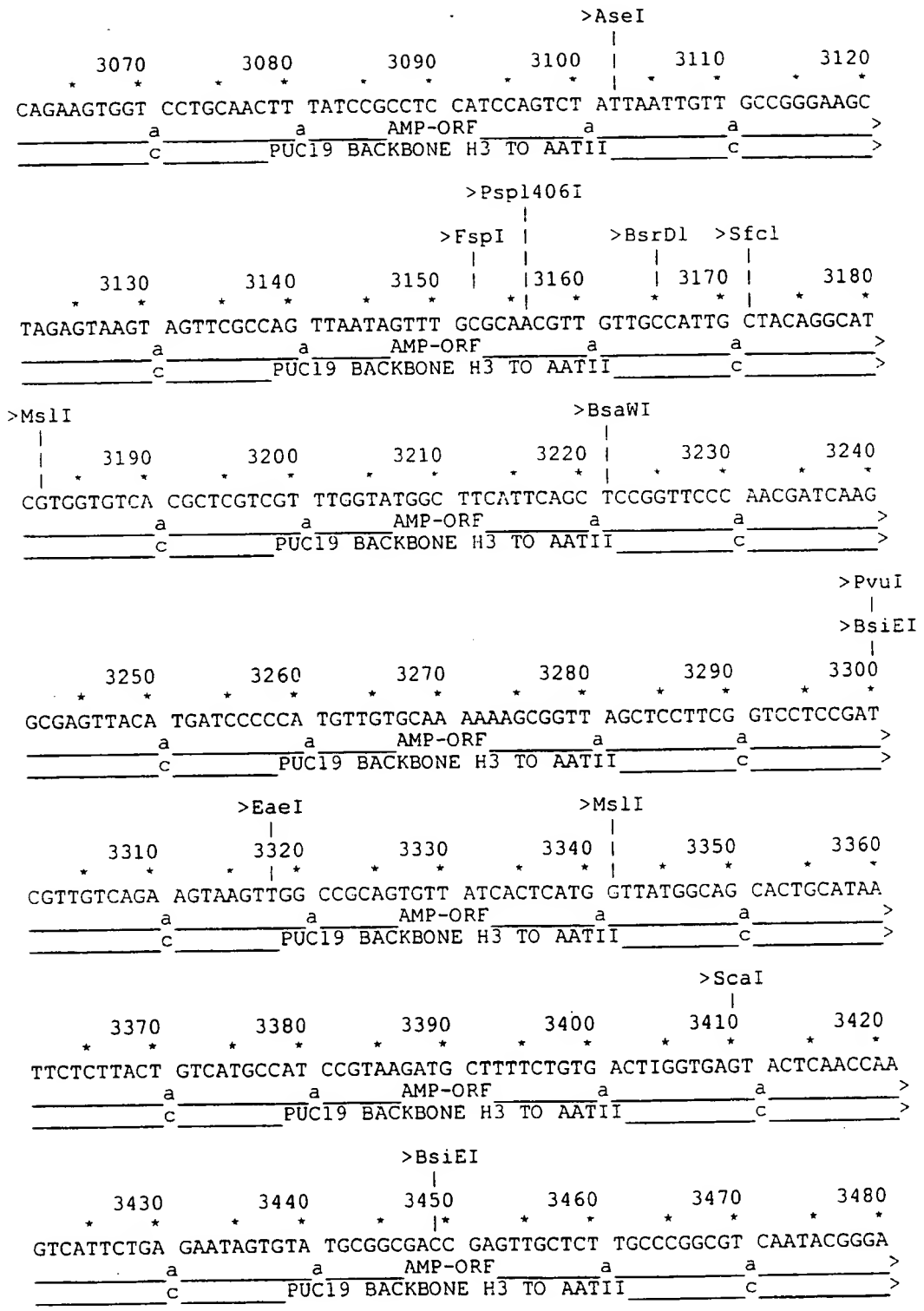

FIG. 4
(CONTINUED)

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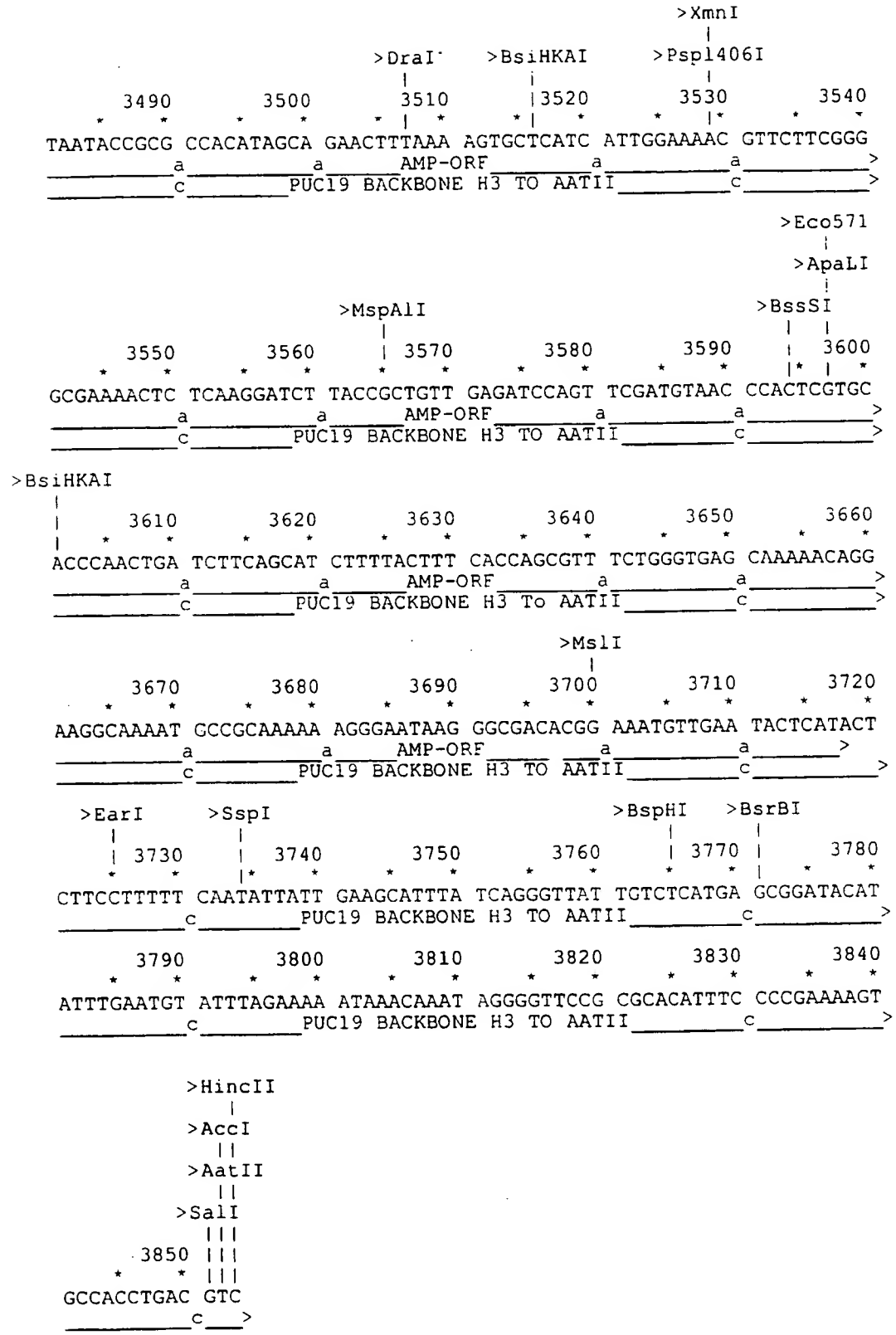


FIG. 4
(CONTINUED)

)OCID: <WO__9821228A1_IA>

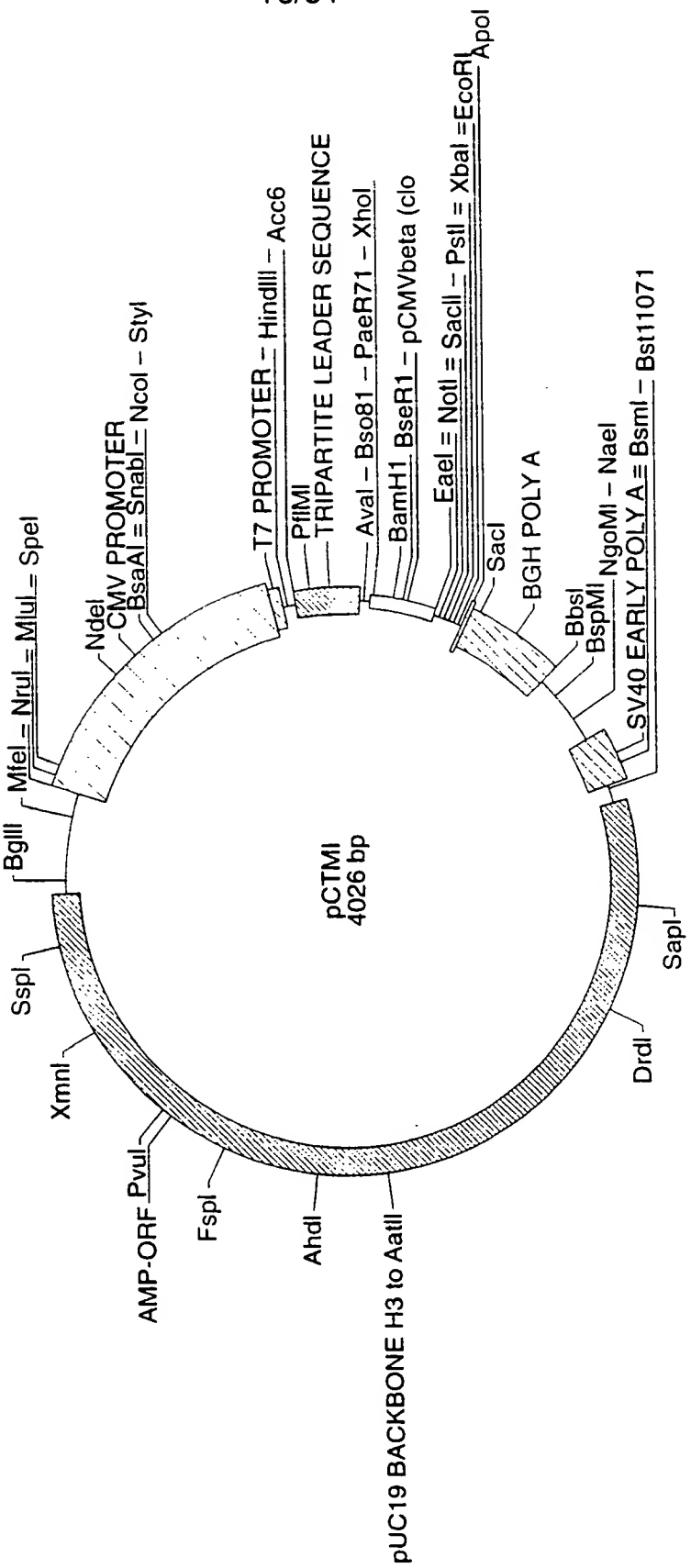
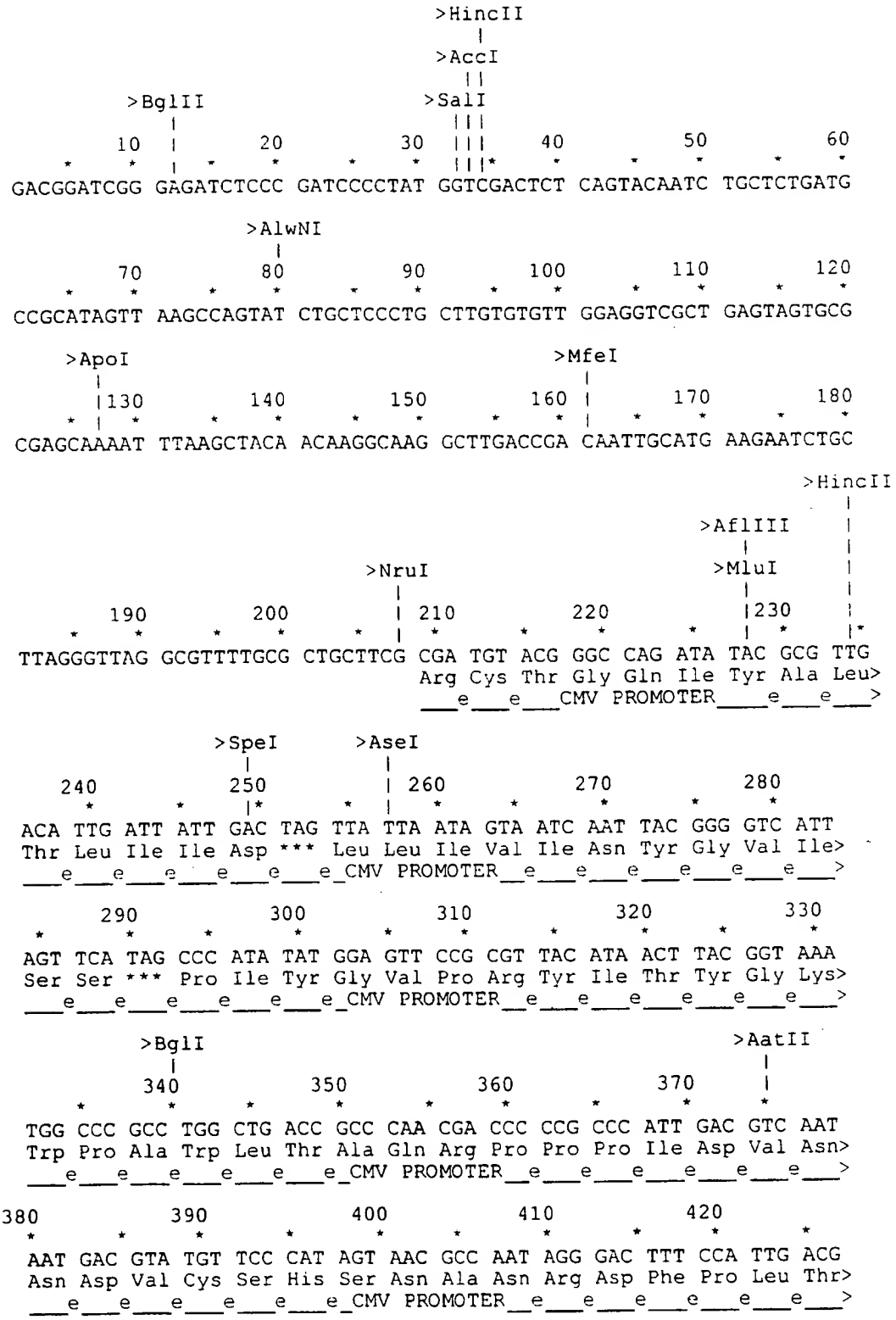


FIG. 5

DOCID: <WO___9821228A1_IA>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>BglII
>SalI
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>AlwNI
70 80 90 100 110 120
CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
>ApoI
>MfeI
130 140 150 160 170 180
CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
>HincII
>AflIII
>NruI
>MluI
190 200 210 220 230
TTAGGGTTAG GCGTTTTGCG CTGCTTCG CGA TGT ACG GGC CAG ATA TAC GCG TTG
Arg Cys Thr Gly Gln Ile Tyr Ala Leu>
___e___e___CMV PROMOTER____e___e___>
>SpeI
>AseI
240 250 260 270 280
ACA TTG ATT ATT GAC TAG TTA TTA ATA GTA ATC AAT TAC GGG GTC ATT
Thr Leu Ile Ile Asp *** Leu Leu Ile Val Ile Asn Tyr Gly Val Ile>
___e___e___e___e___e___e_CMV PROMOTER__e___e___e___e___e___e___>
290 300 310 320 330
AGT TCA TAG CCC ATA TAT GGA GTT CCG CGT TAC ATA ACT TAC GGT AAA
Ser Ser *** Pro Ile Tyr Gly Val Pro Arg Tyr Ile Thr Tyr Gly Lys>
___e___e___e___e___e___e_CMV PROMOTER__e___e___e___e___e___e___>
>BglI
>AatII
340 350 360 370
TGG CCC GCC TGG CTG ACC GCC CAA CGA CCC CCG CCC ATT GAC GTC AAT
Trp Pro Ala Trp Leu Thr Ala Gln Arg Pro Pro Pro Ile Asp Val Asn>
___e___e___e___e___e___e___e_CMV PROMOTER__e___e___e___e___e___e___>
380 390 400 410 420
AAT GAC GTA TGT TCC CAT AGT AAC GCC AAT AGG GAC TTT CCA TTG ACG
Asn Asp Val Cys Ser His Ser Asn Ala Asn Arg Asp Phe Pro Leu Thr>
___e___e___e___e___e___e___e_CMV PROMOTER__e___e___e___e___e___e___>
FIG. 6

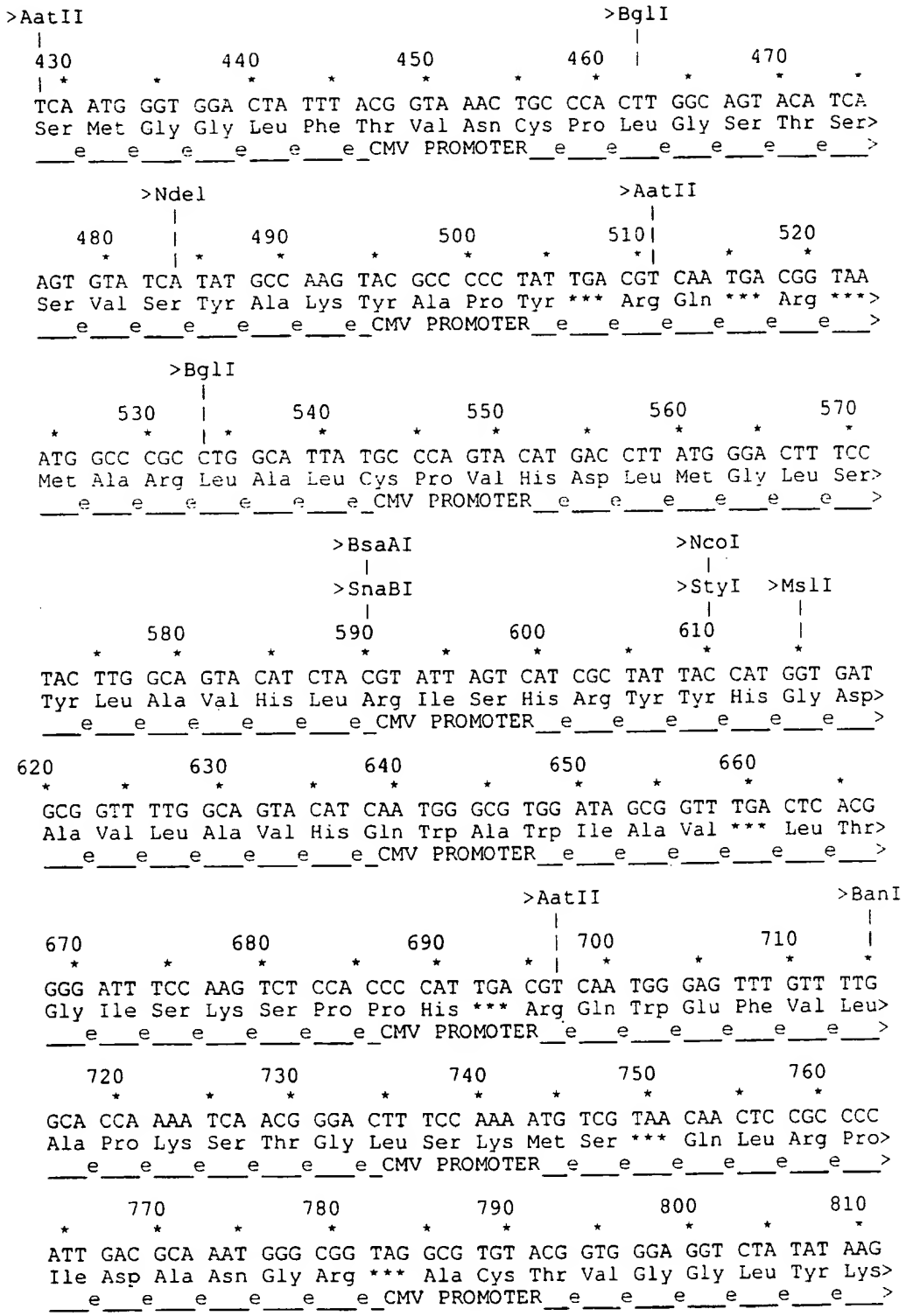

FIG. 6
(CONTINUED)

3DOCID: <WO___9821228A1_IA>

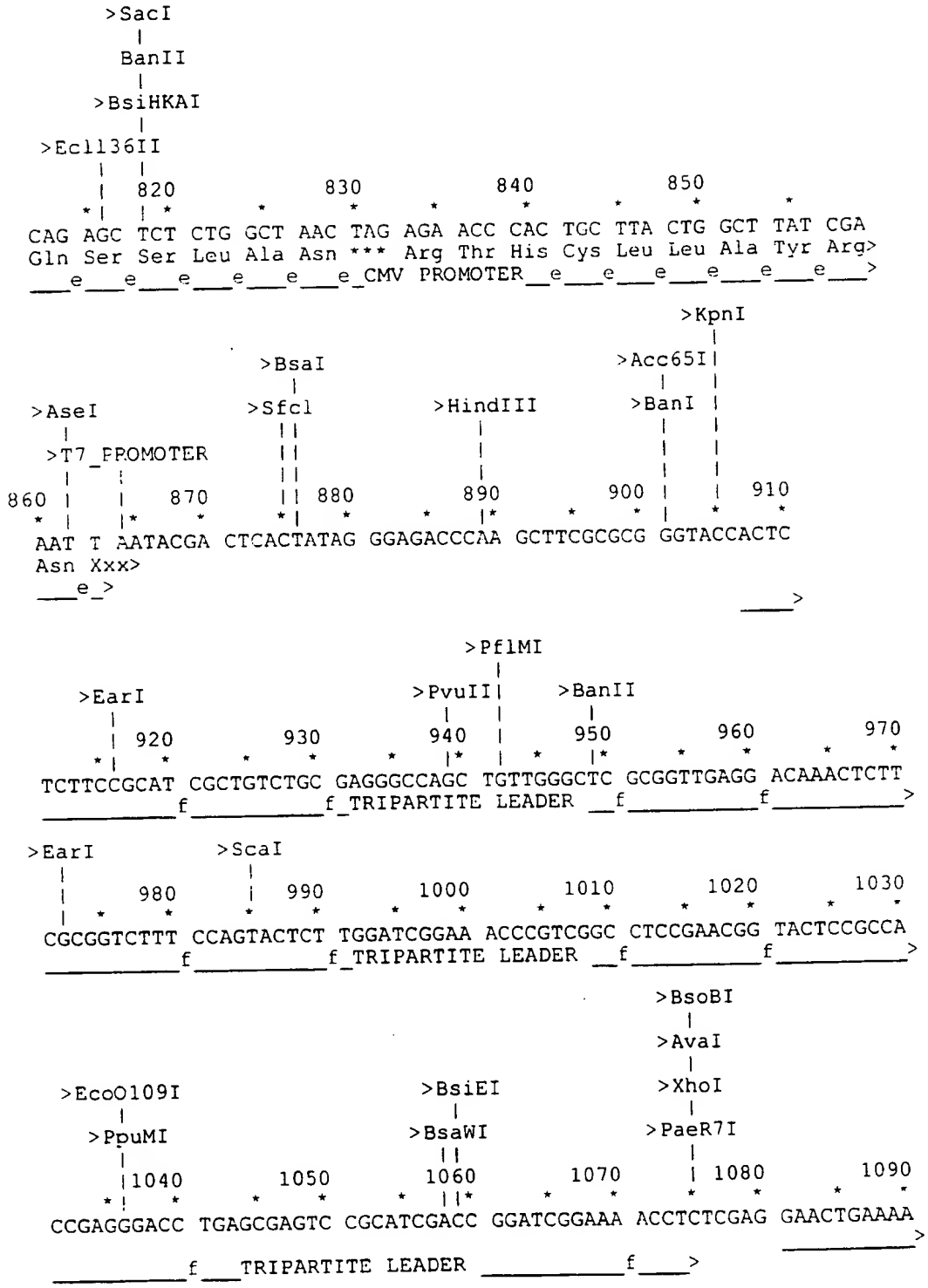

FIG. 6
(CONTINUED)

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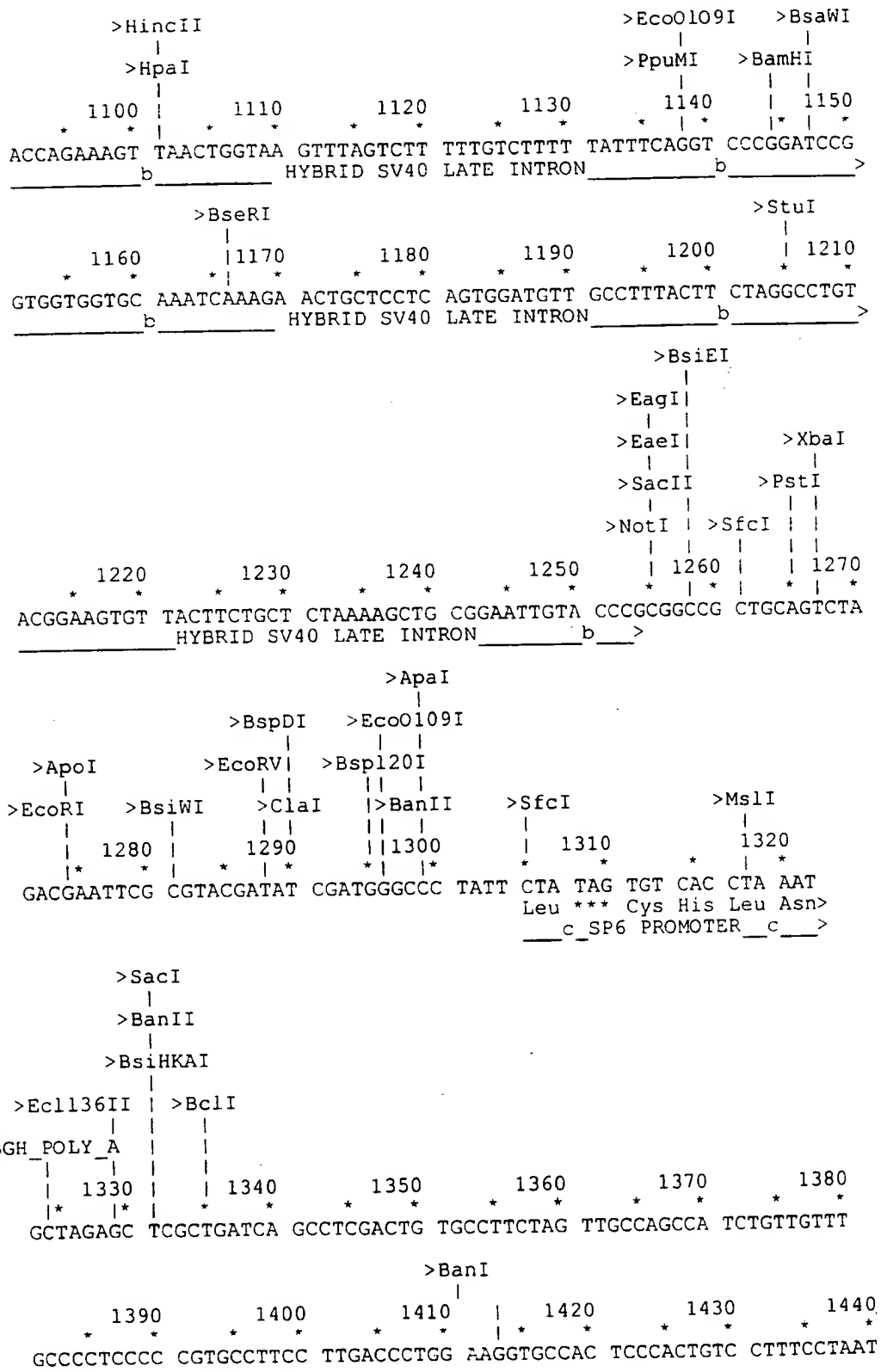

FIG. 6
(CONTINUED)

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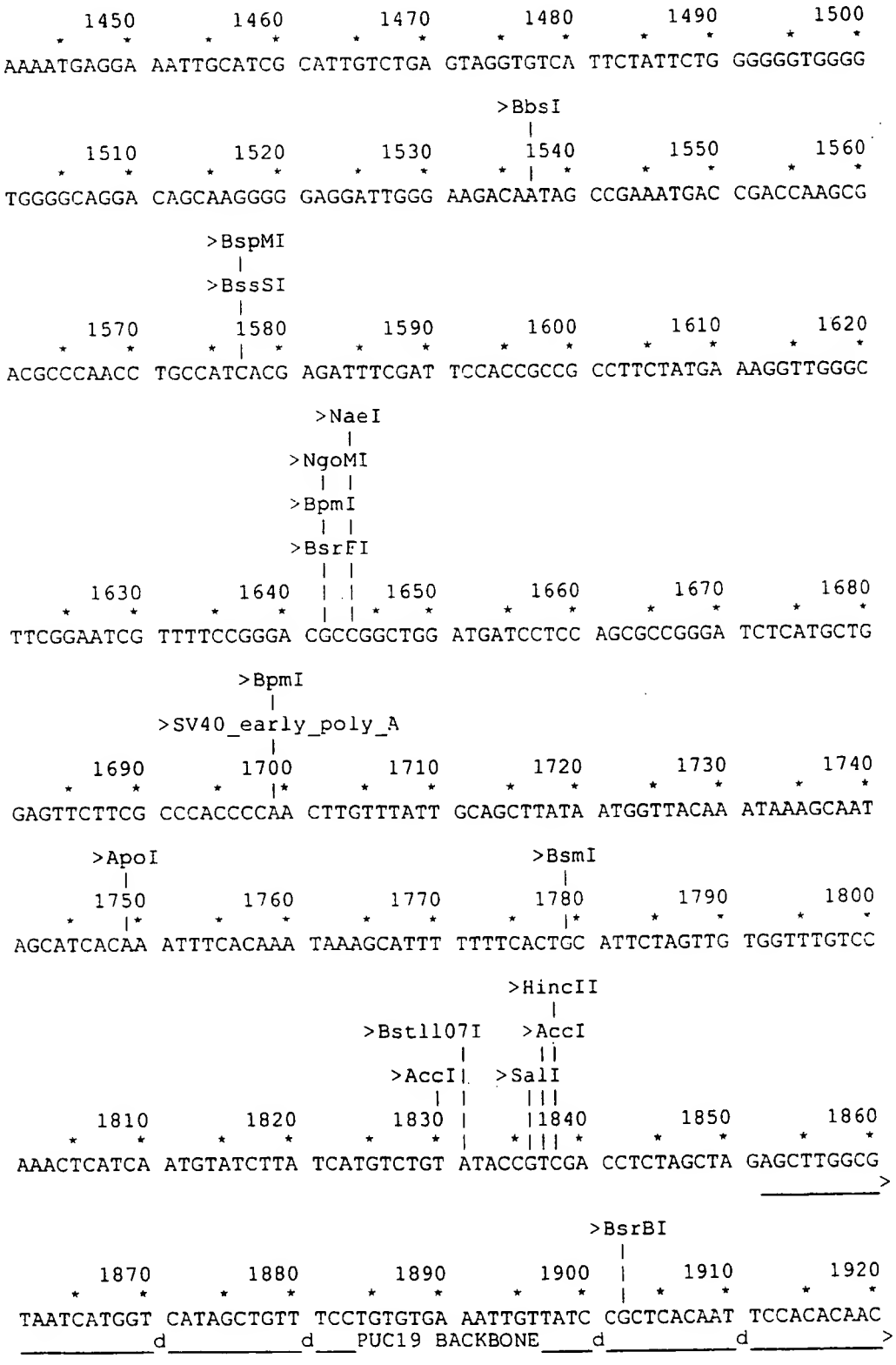

FIG. 6
(CONTINUED)

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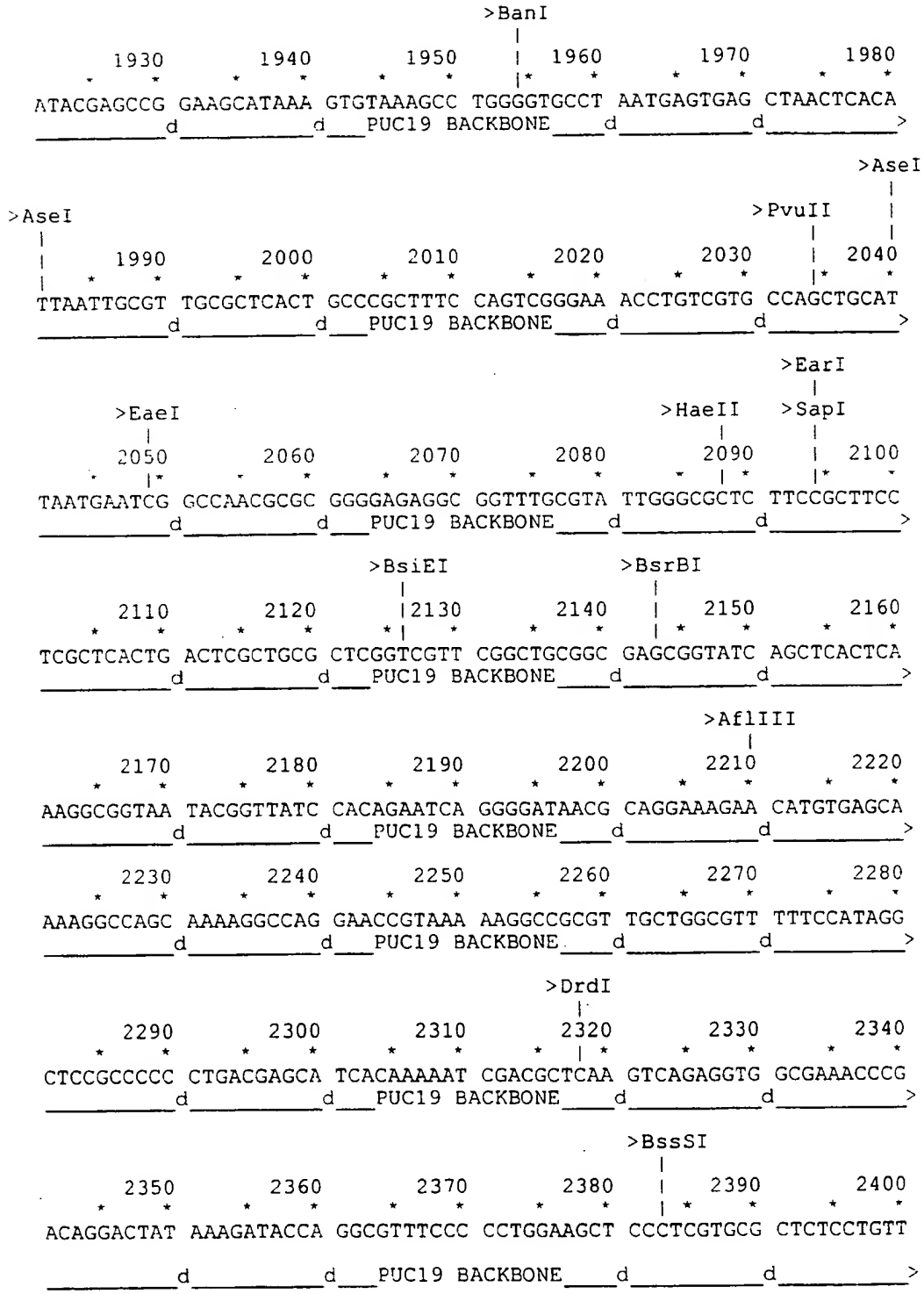

FIG. 6
(CONTINUED)

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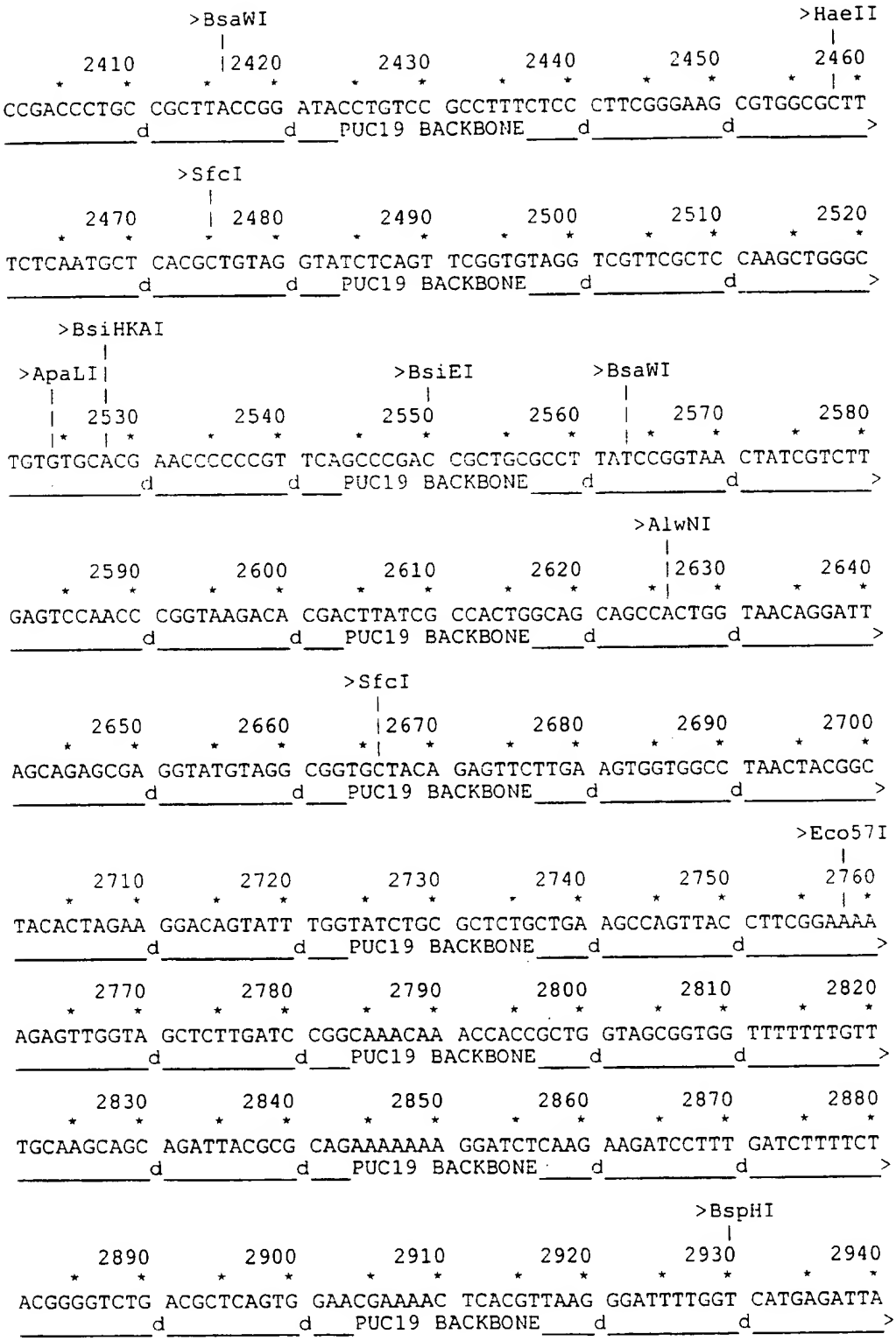

FIG. 6
(CONTINUED)

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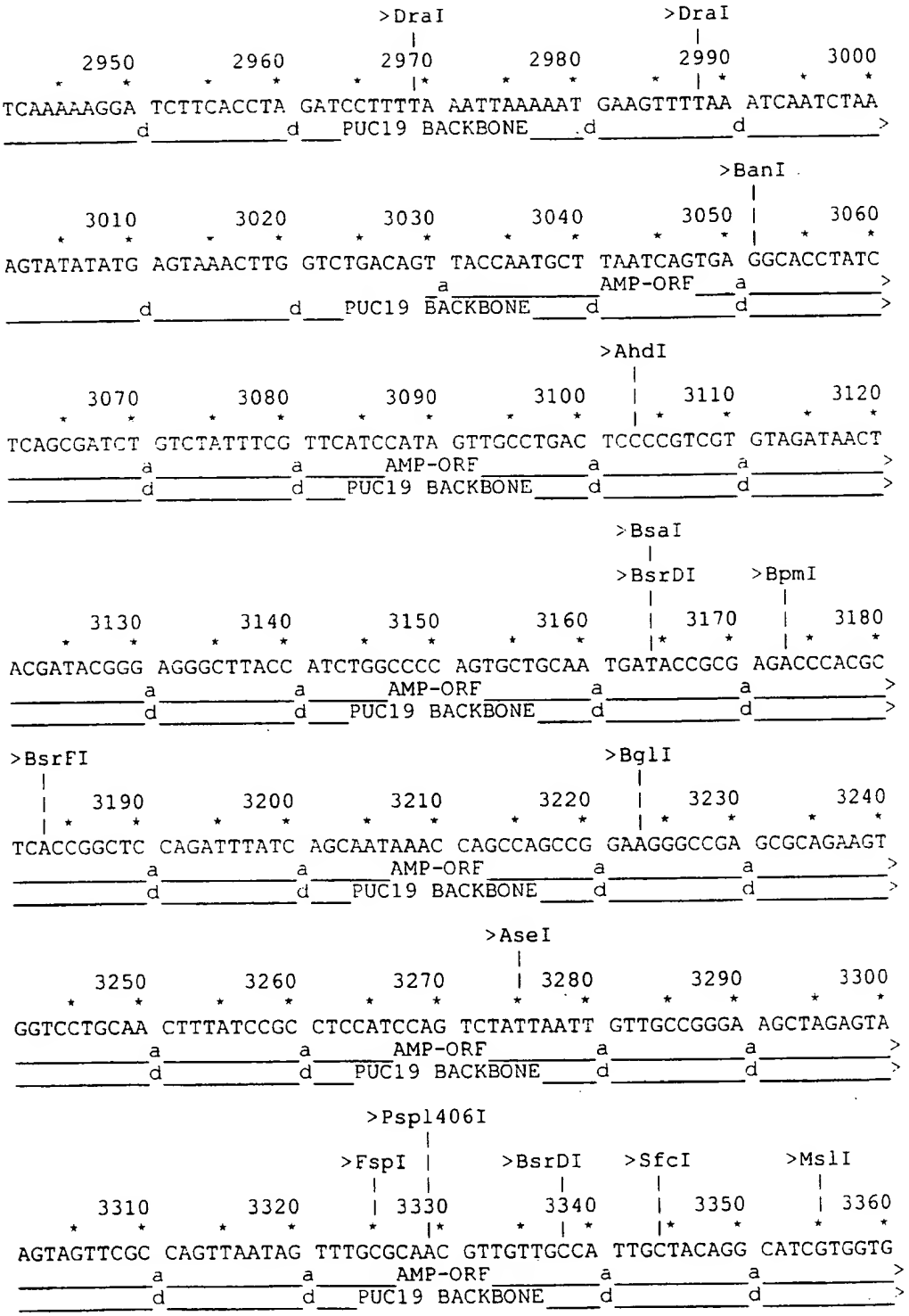

FIG. 6
(CONTINUED)

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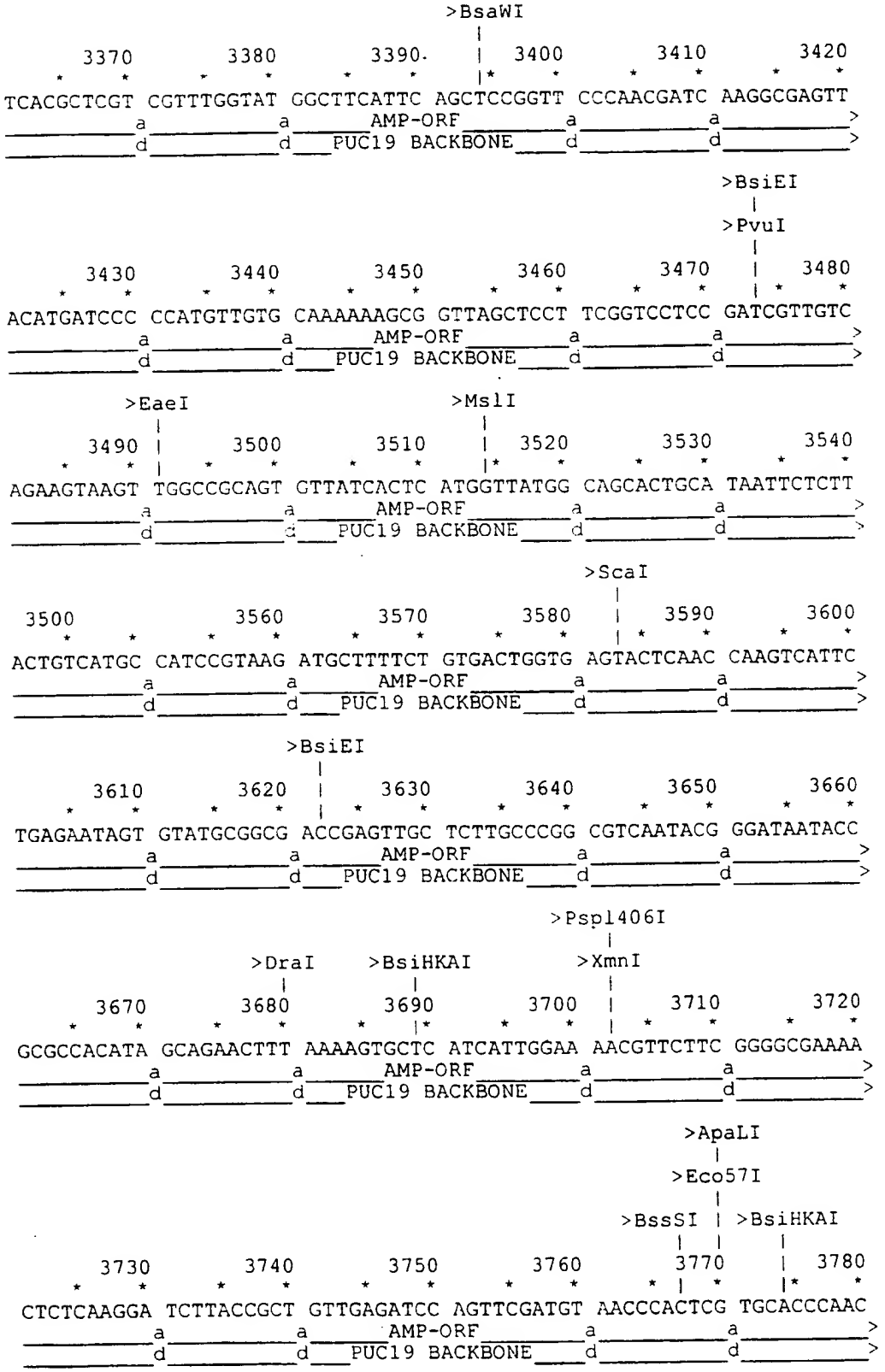

FIG. 6
(CONTINUED)

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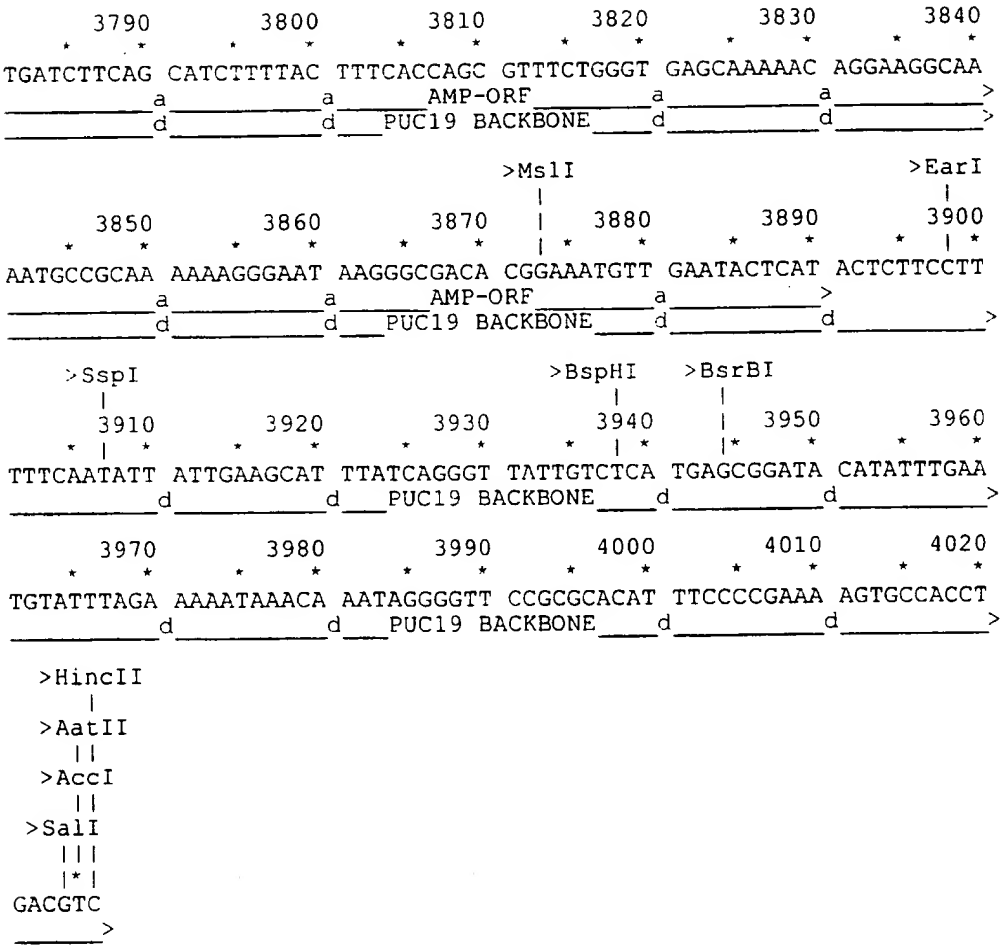

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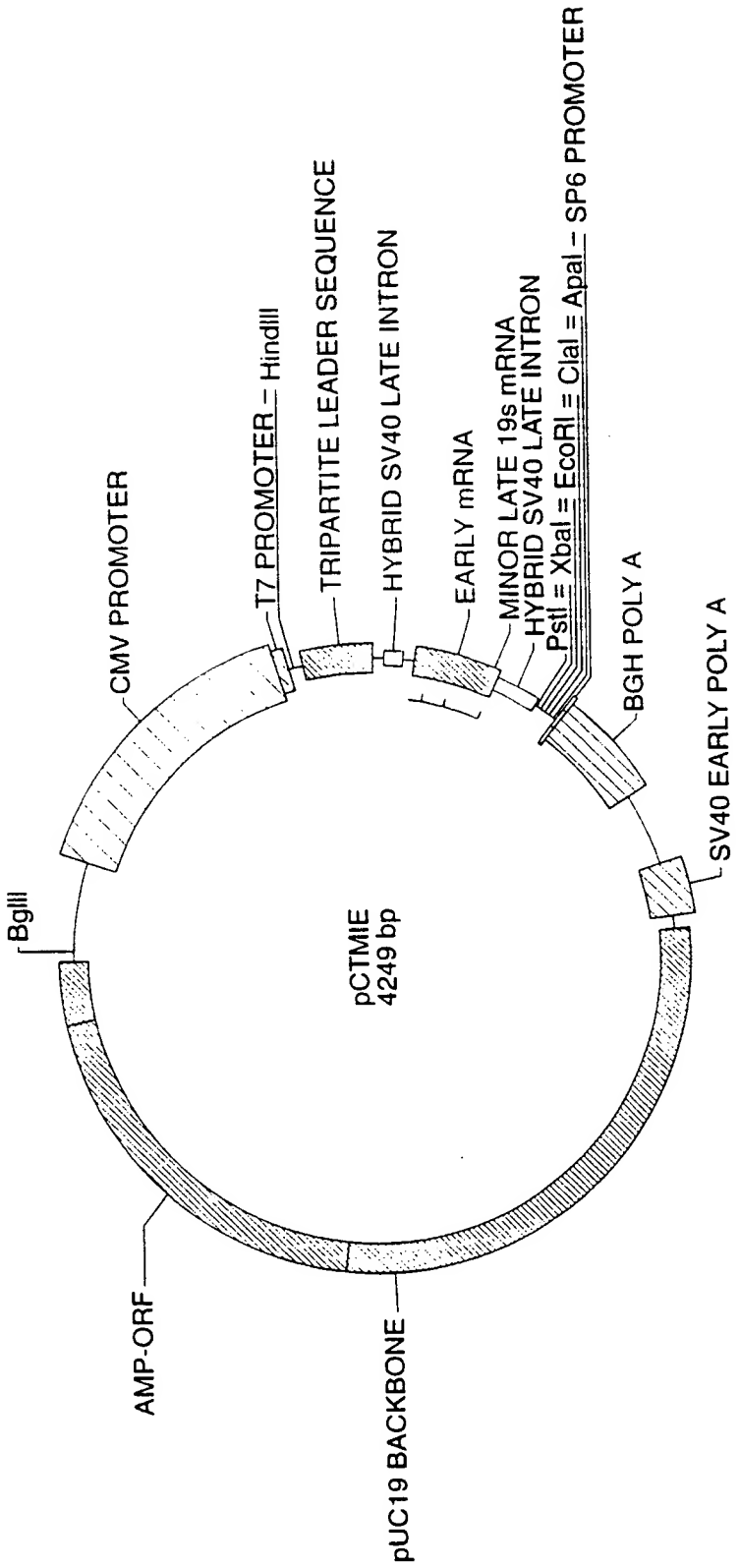

FIG. 7

DOCID: <WO___9821228A1_IA>

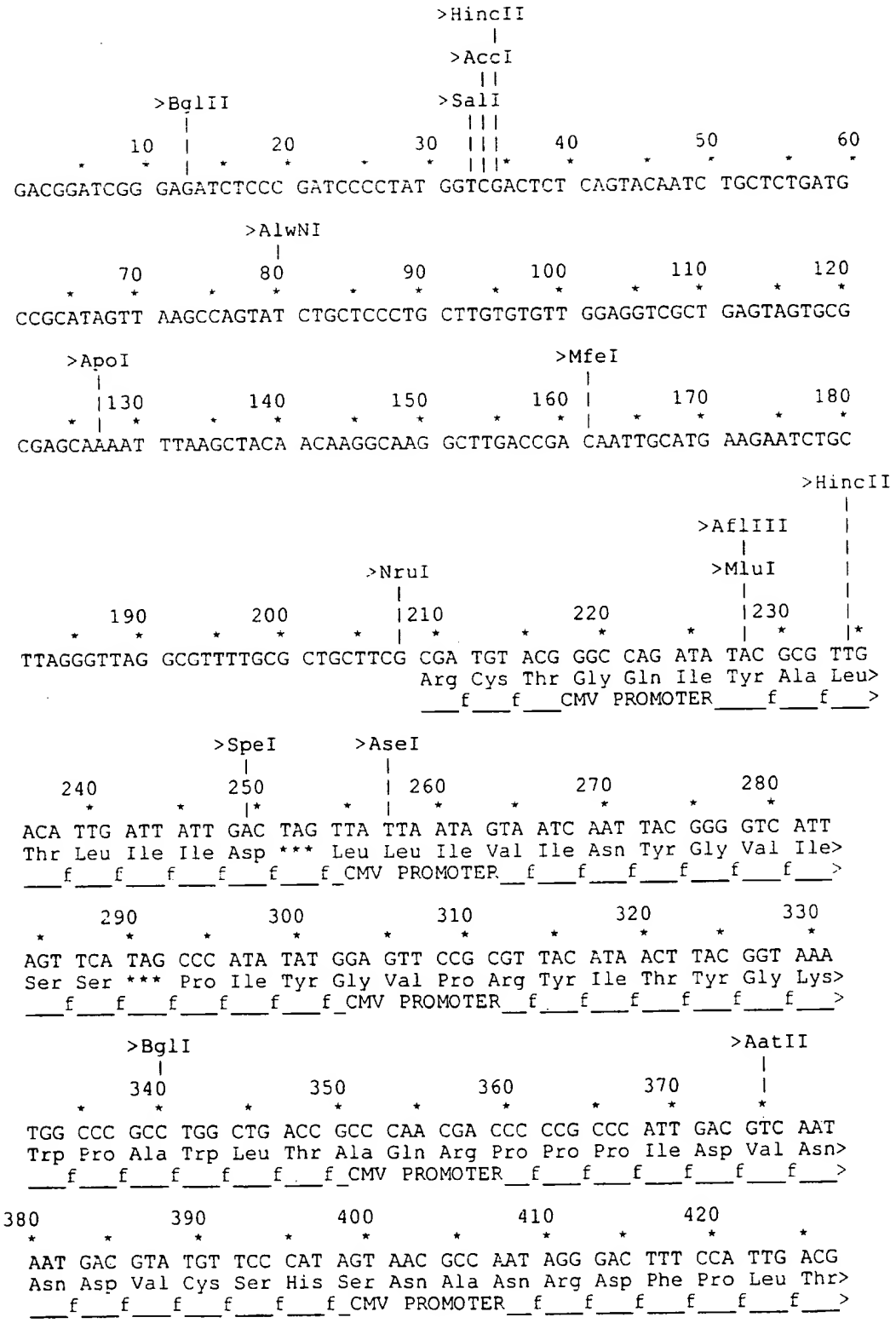

FIG. 8

3DOCID: <WO___9821228A1_IA>

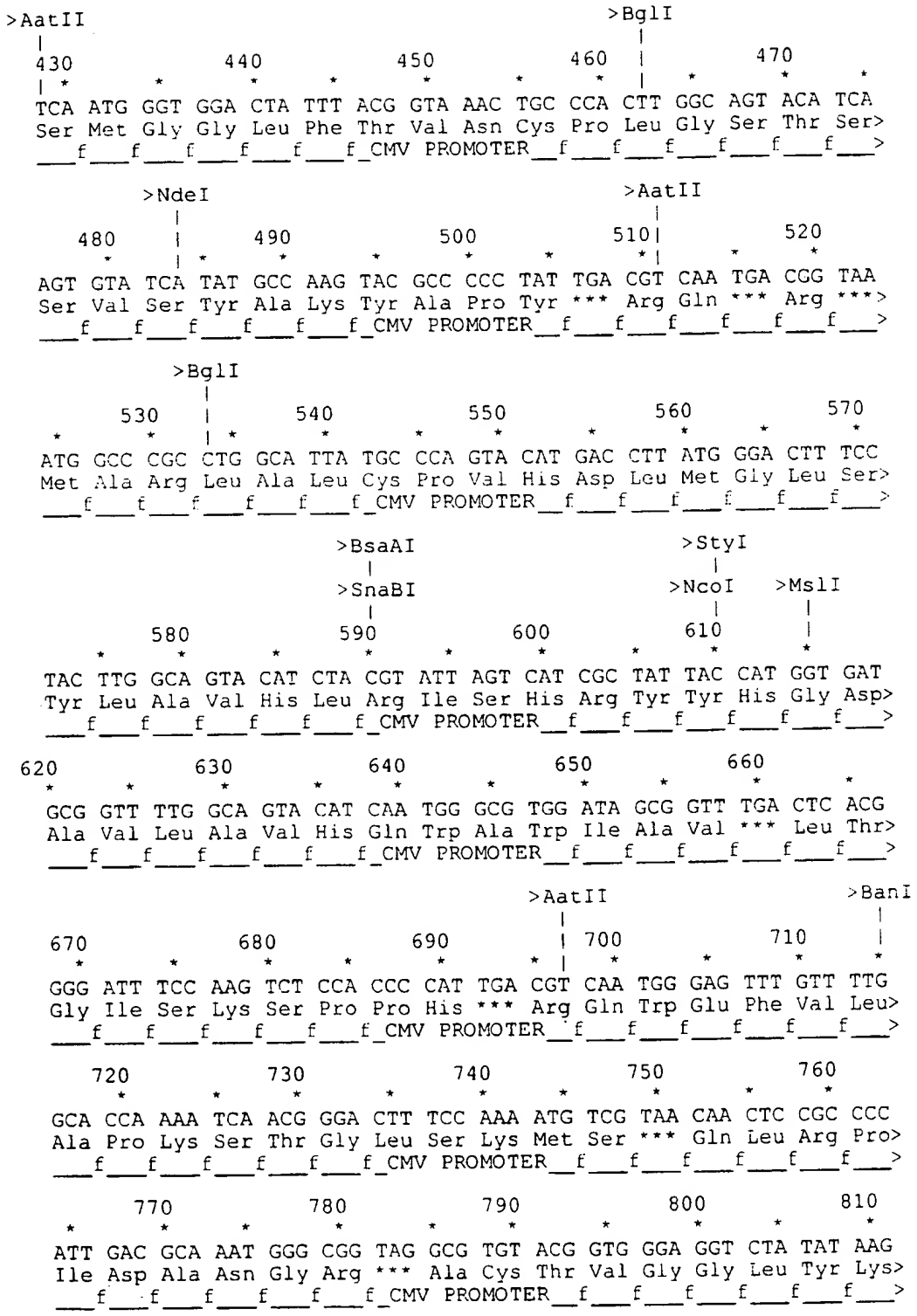

FIG. 8
(CONTINUED)

DOCID: <WO__9821228A1_IA>

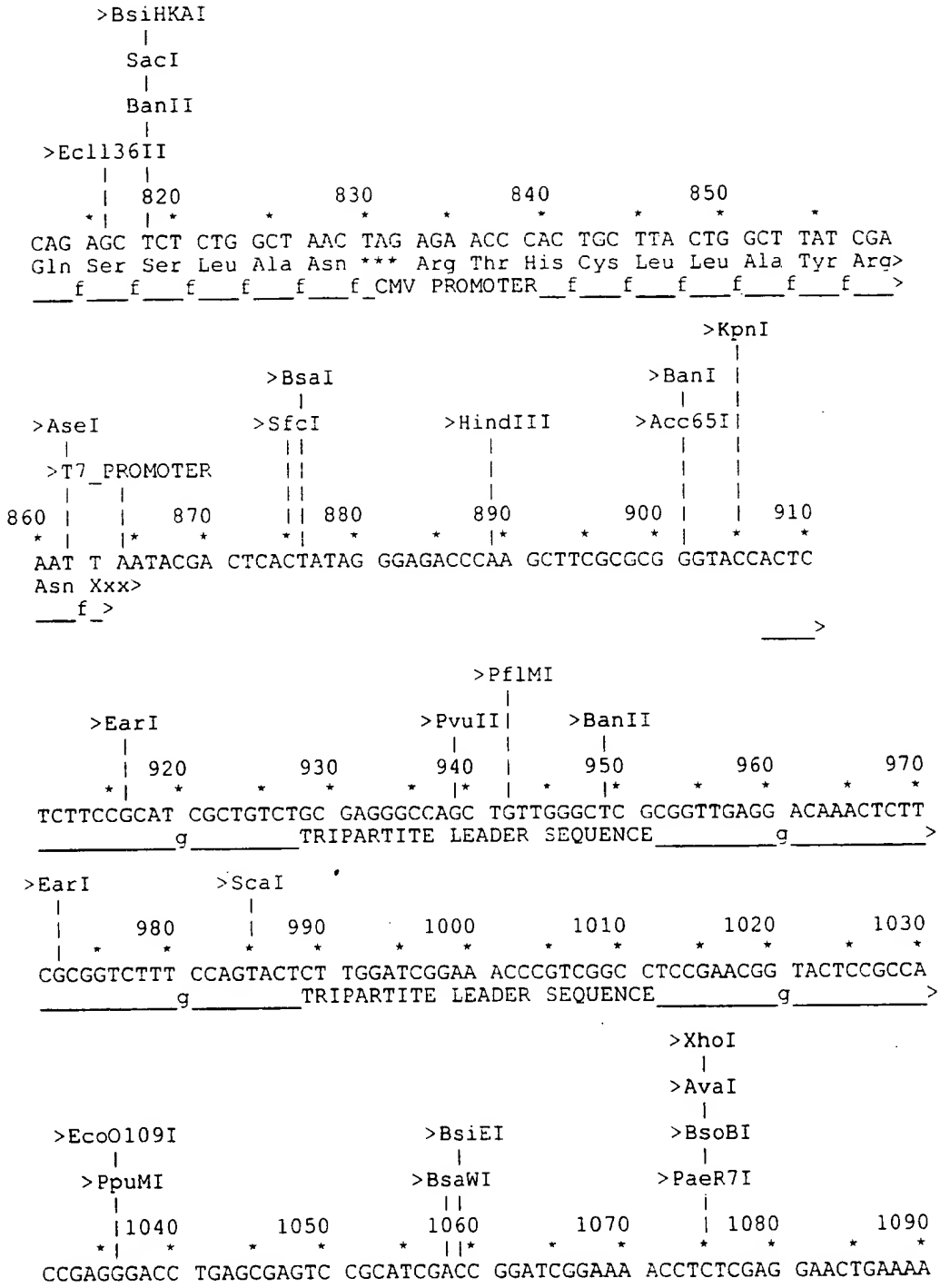

FIG. 8
(CONTINUED)

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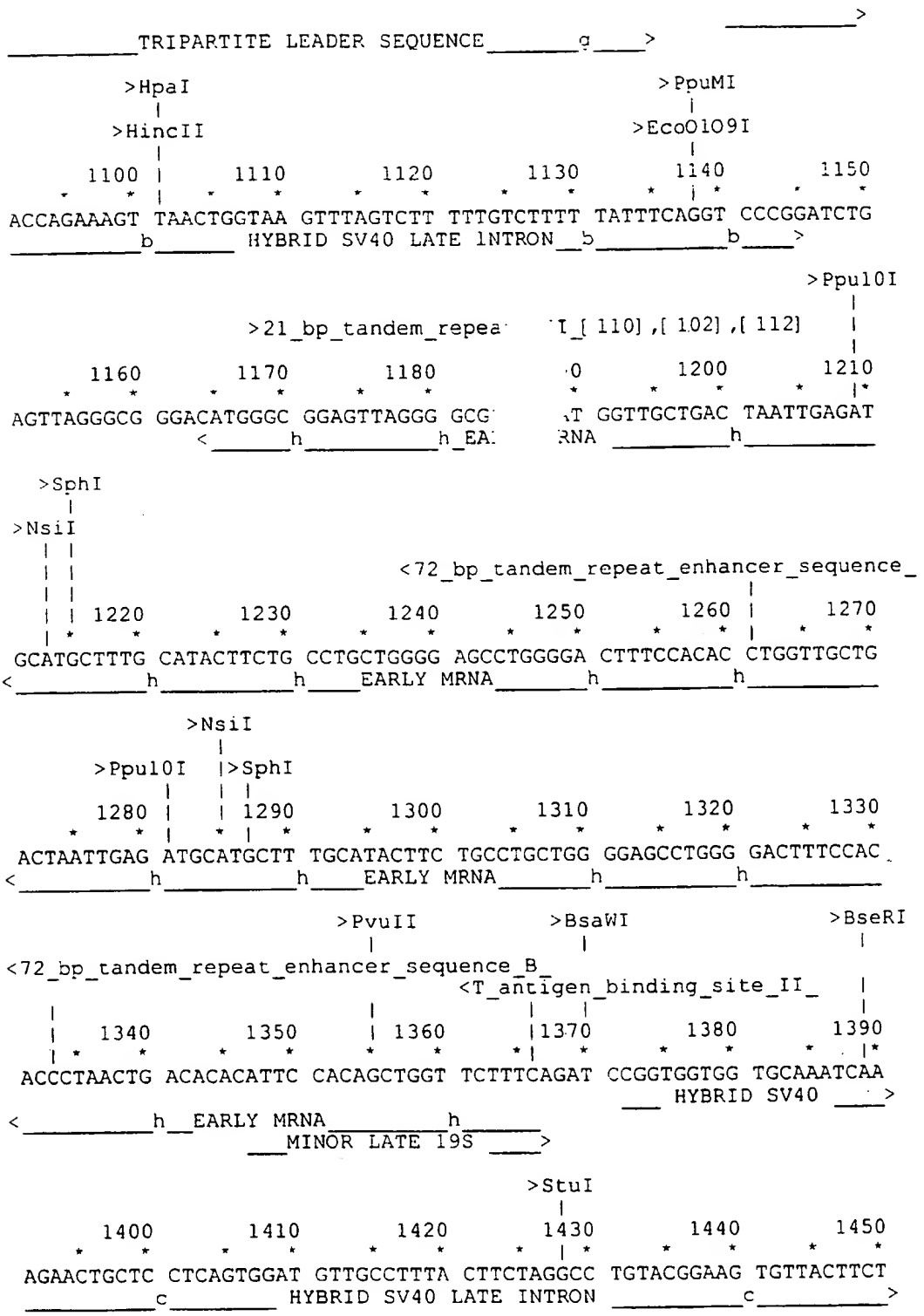

FIG. 8
(CONTINUED)

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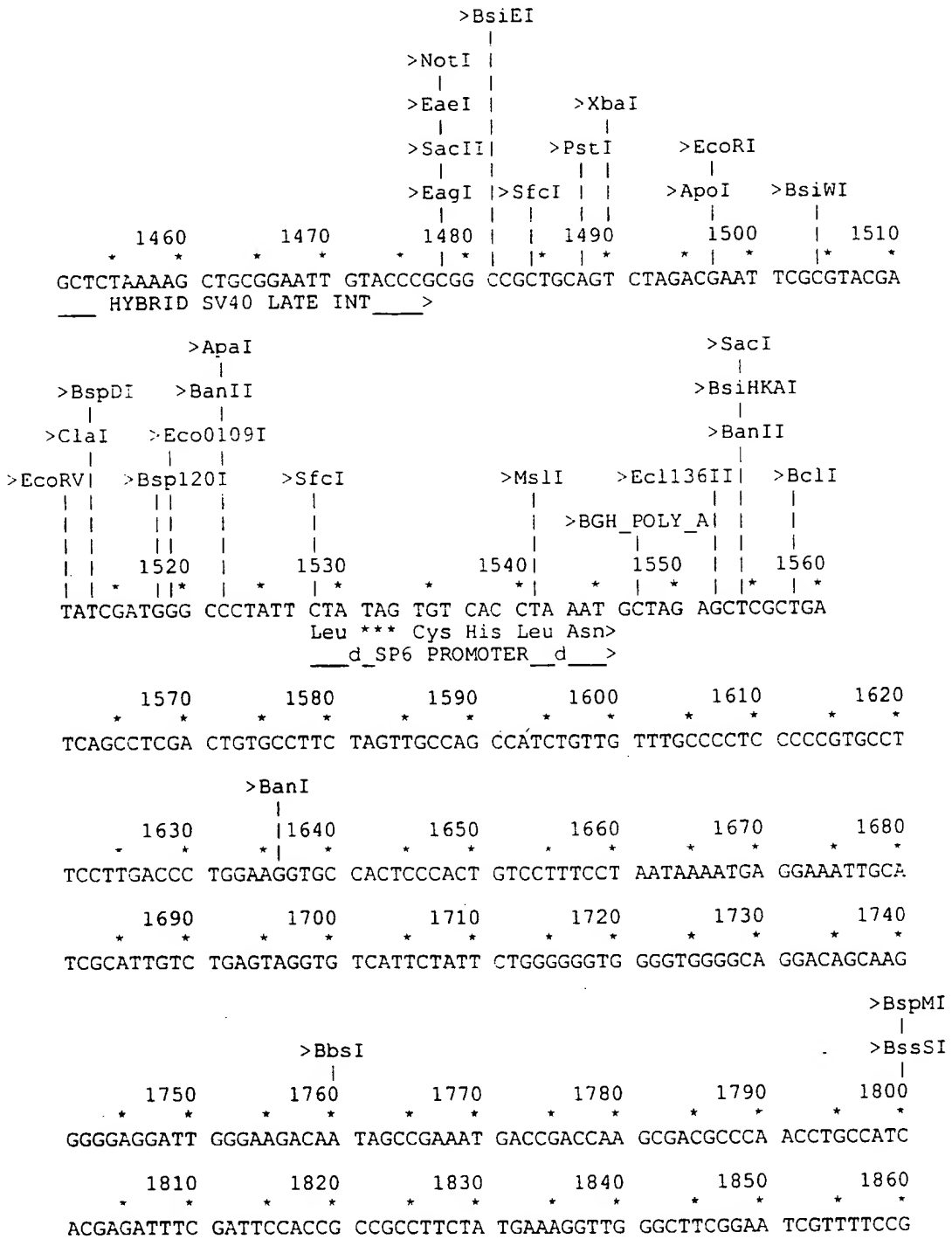

FIG. 8
(CONTINUED)

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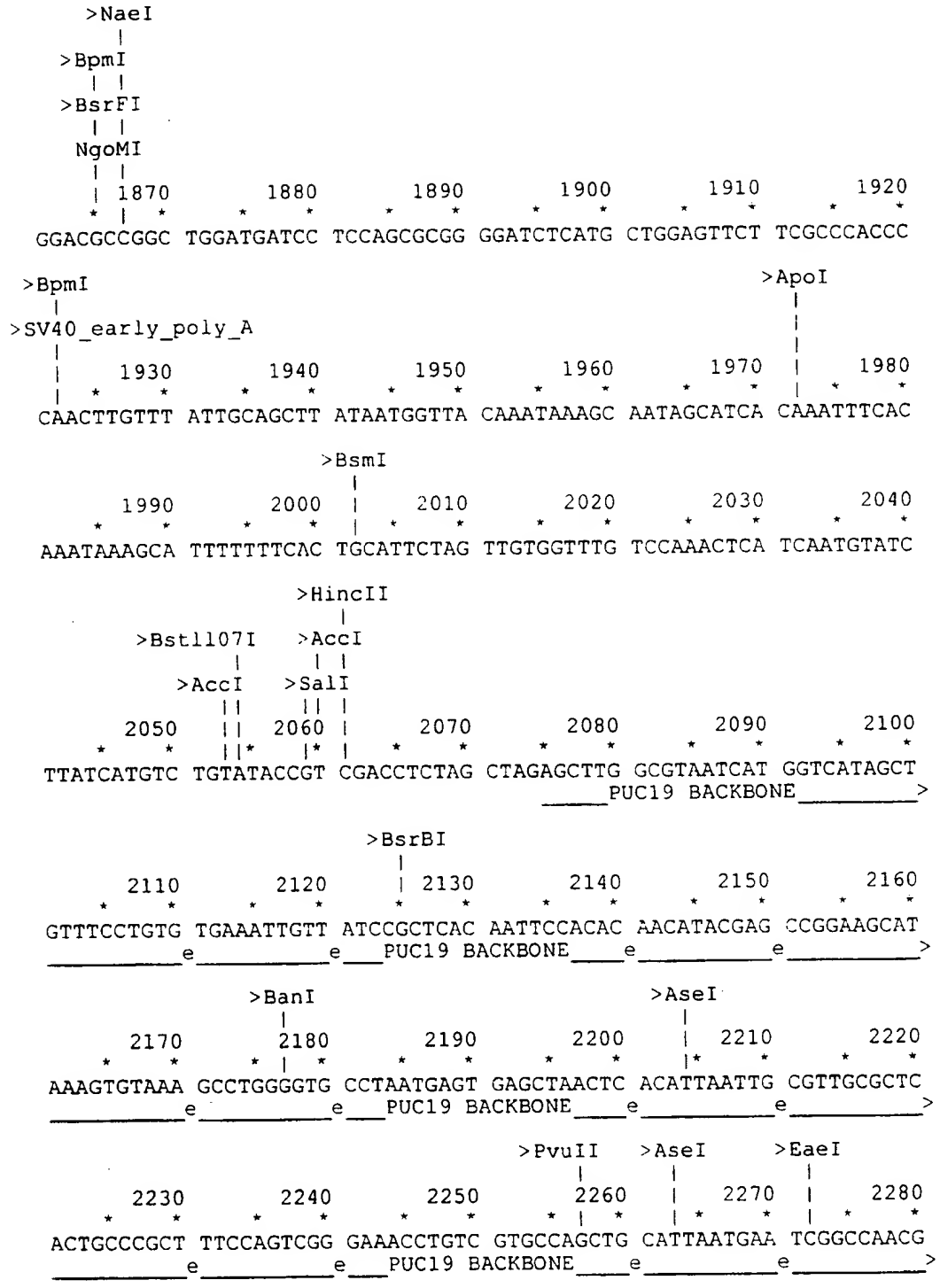

FIG. 8
(CONTINUED)

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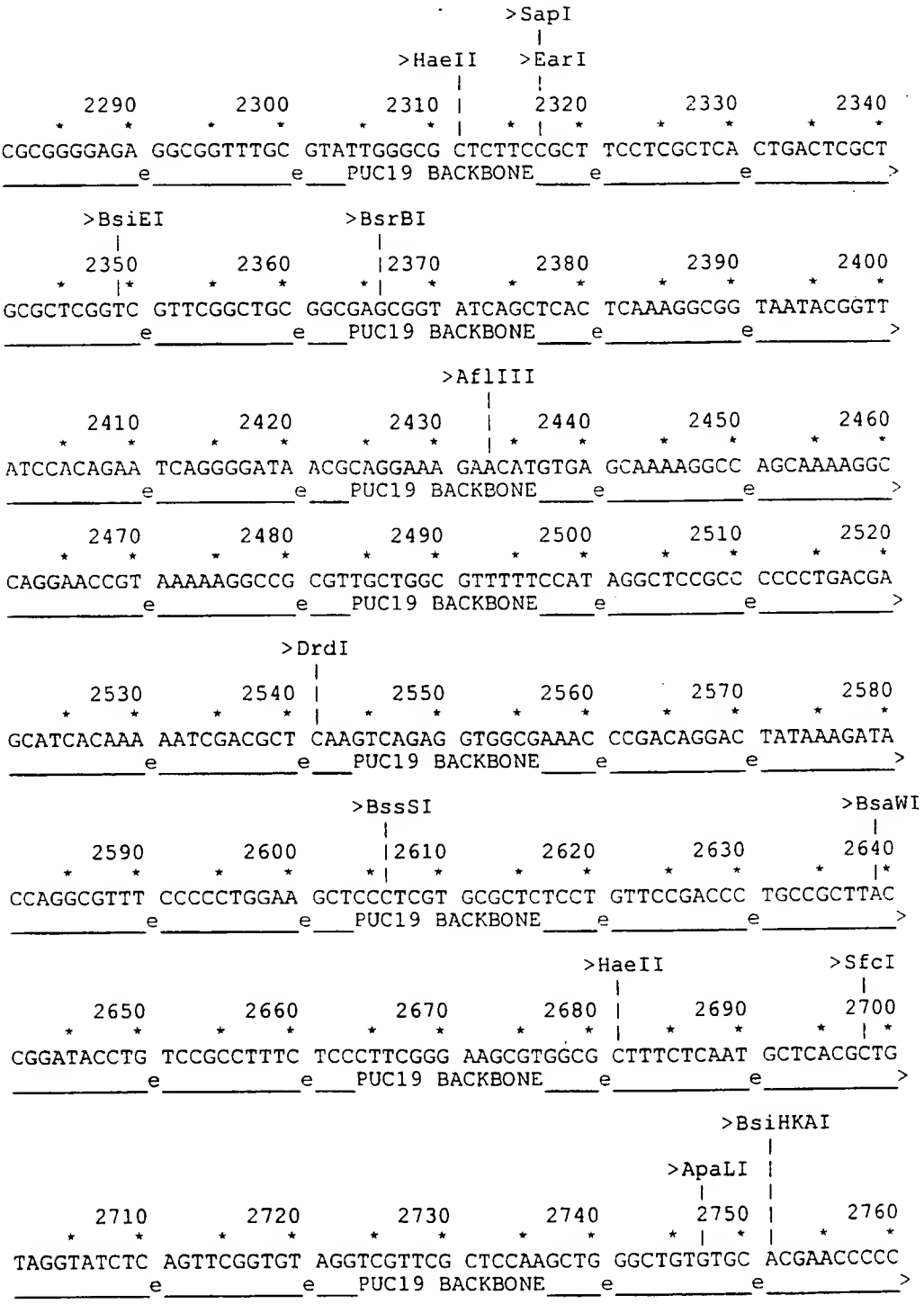

FIG. 8
(CONTINUED)

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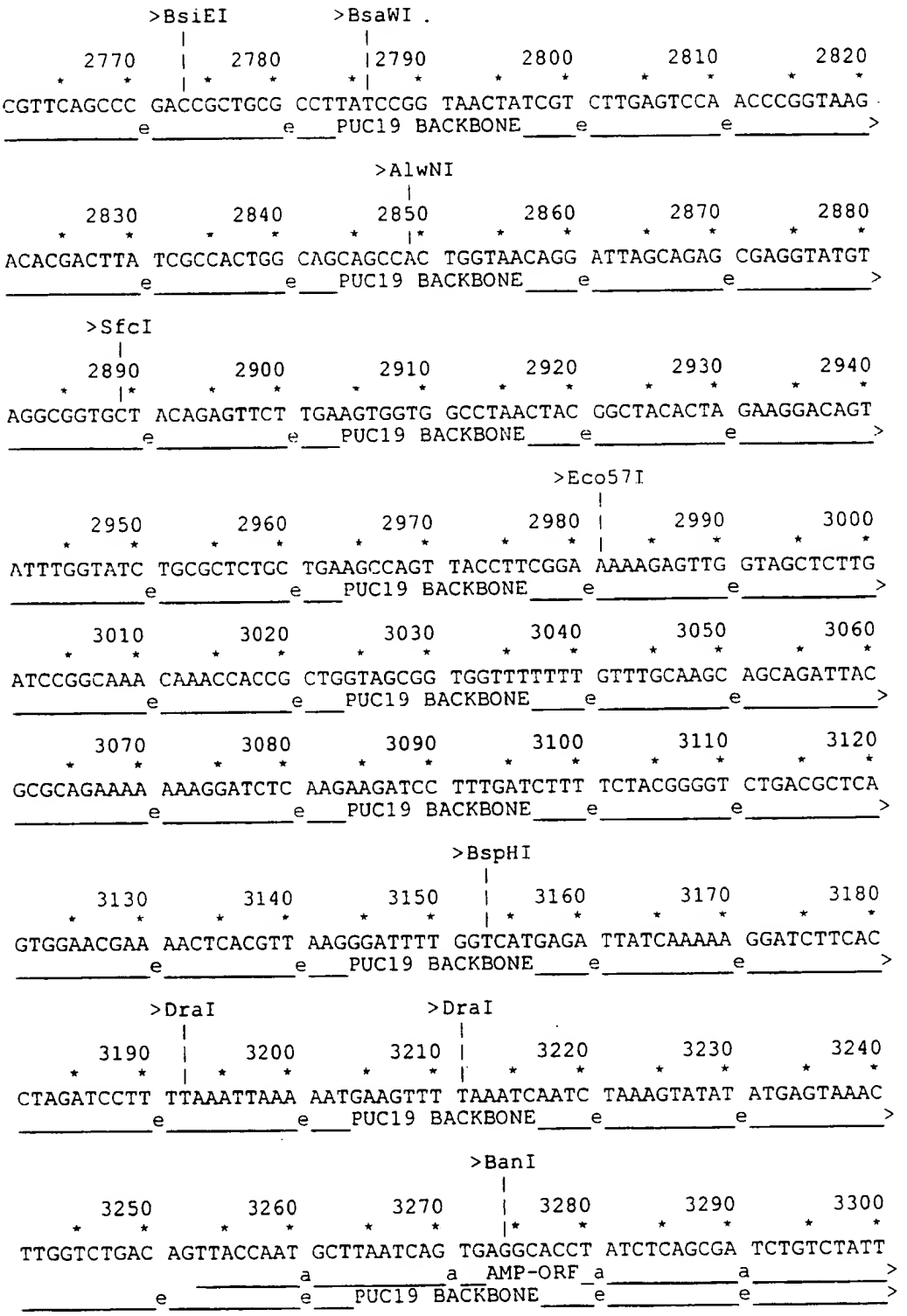

FIG. 8
(CONTINUED)

DOCID: <WO__9821228A1_IA>

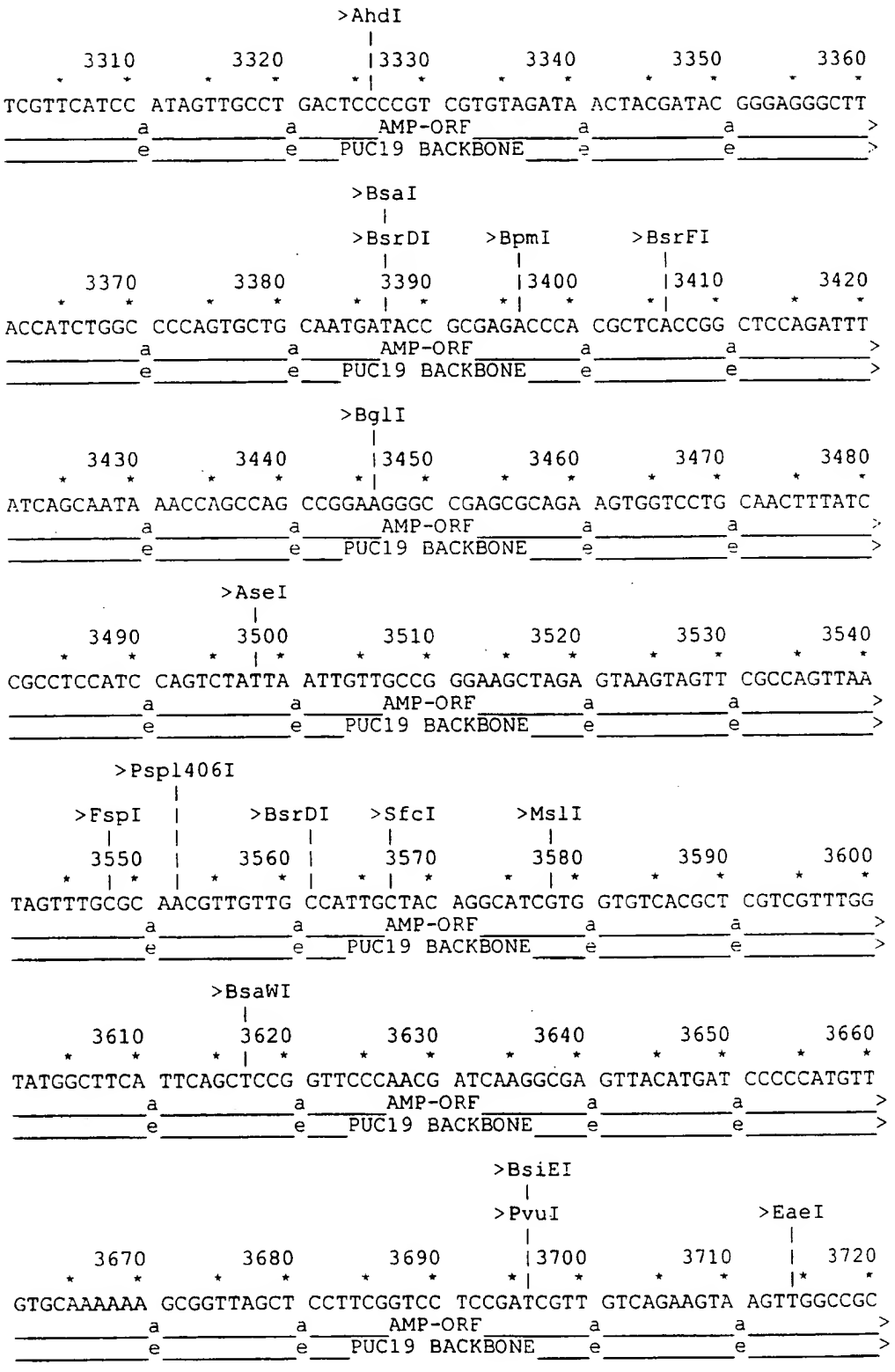

FIG. 8
(CONTINUED)

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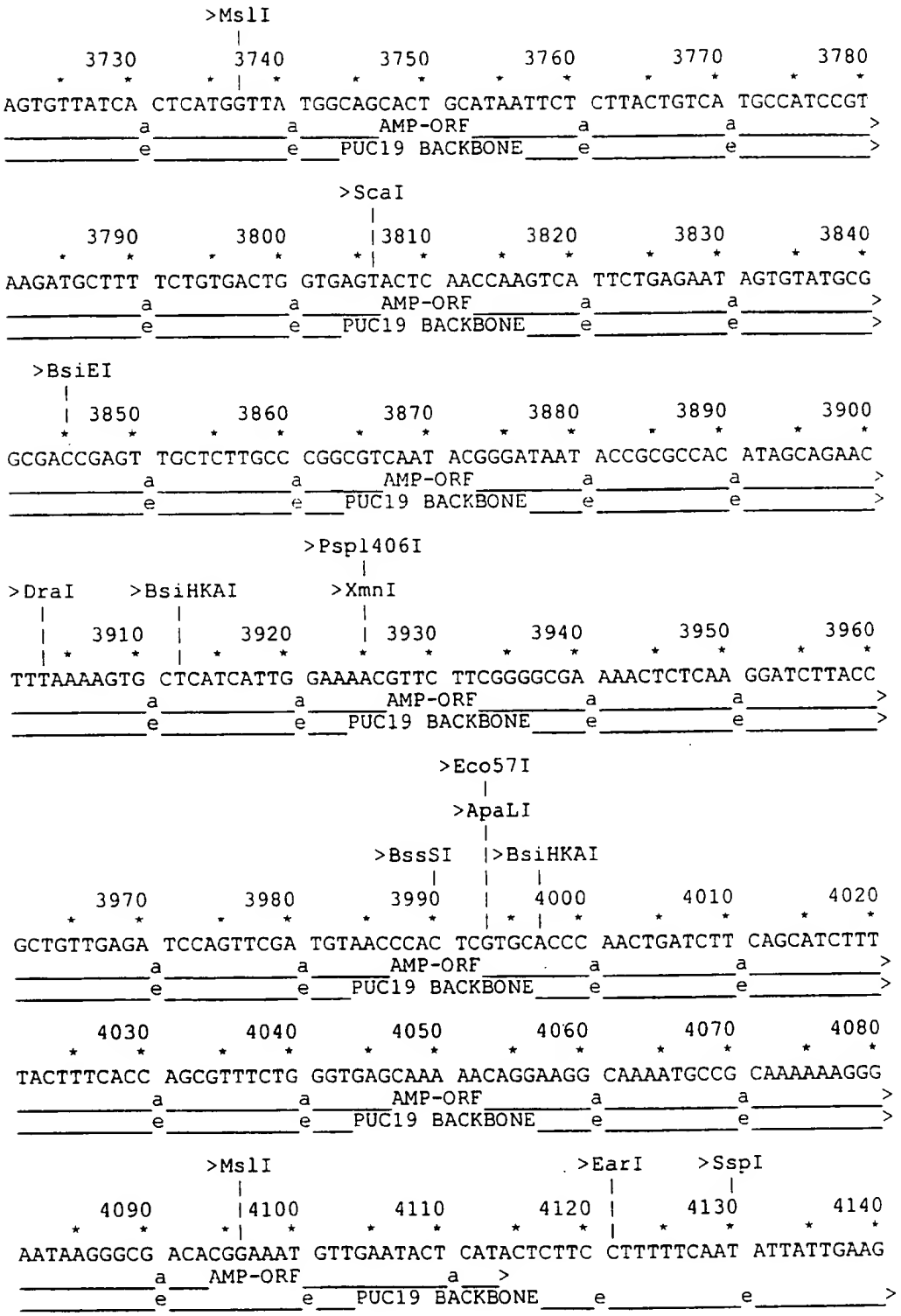

FIG. 8
(CONTINUED)

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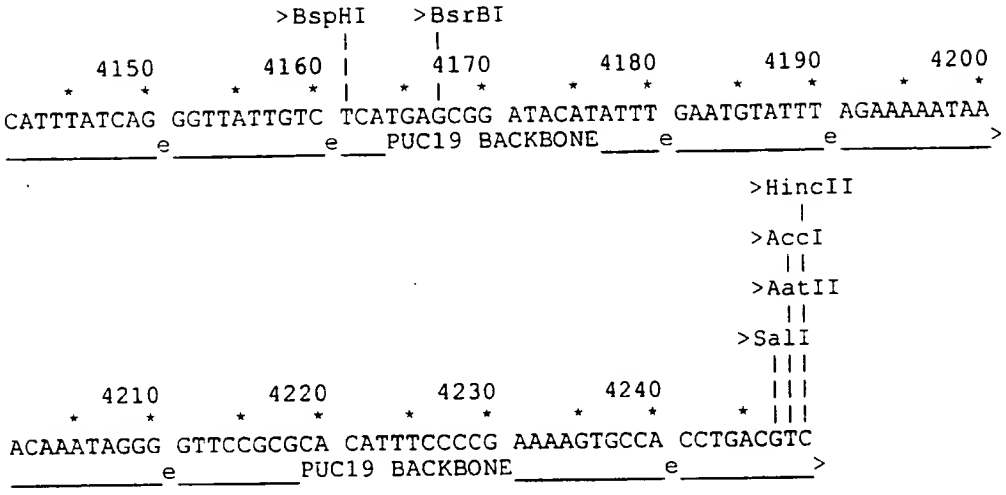

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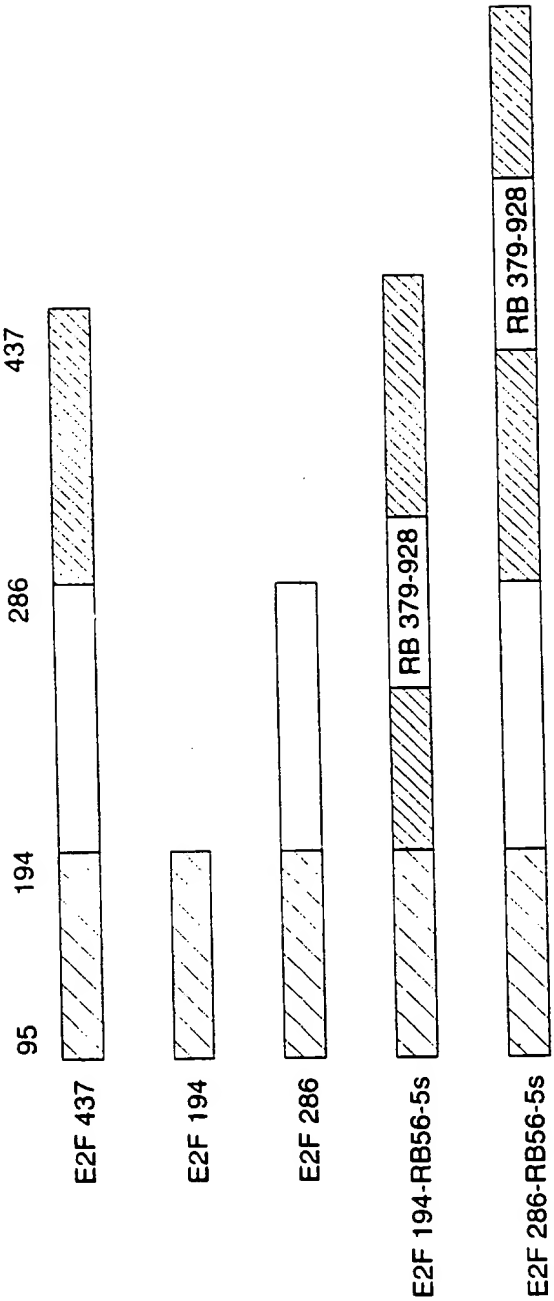


FIG. 9

DOCID: <WO___9821228A1_IA>

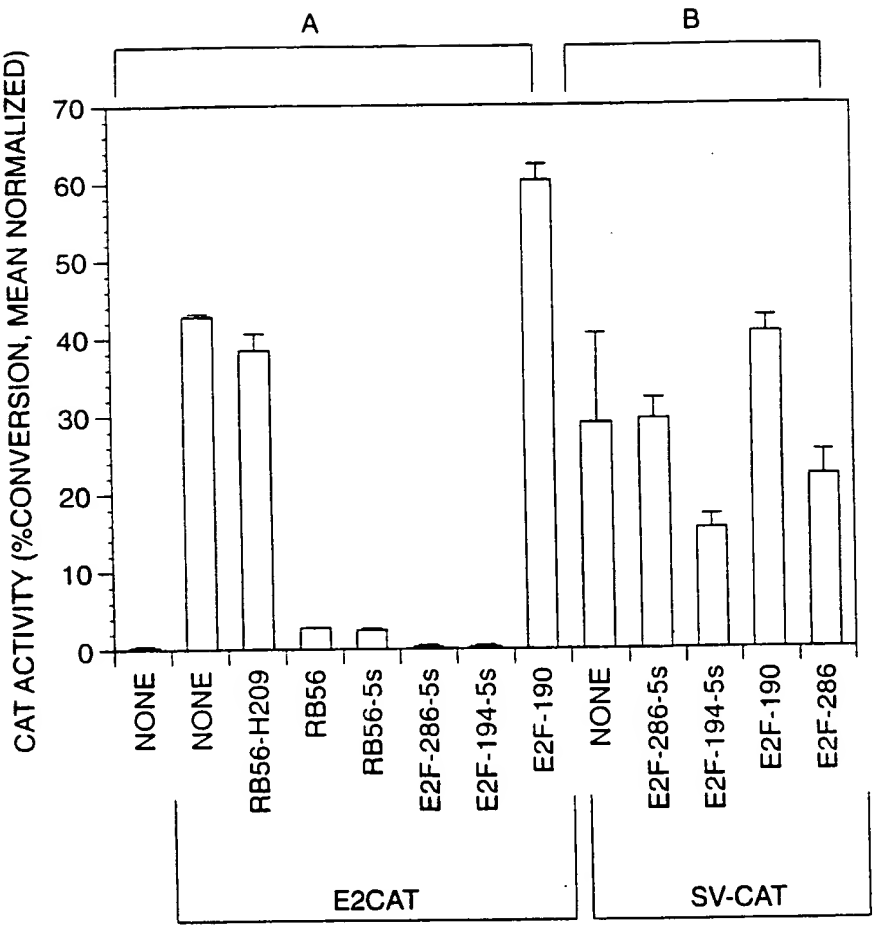


FIG. 10

SDOCID: <WO___9821228A1_IA>

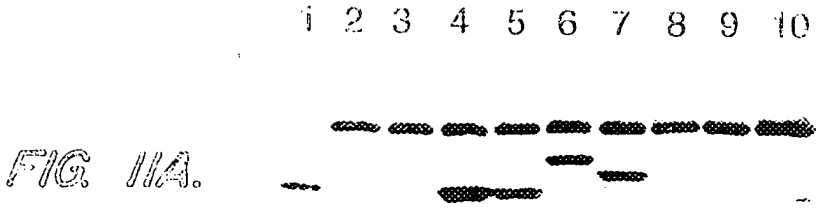


FIG. 11A.

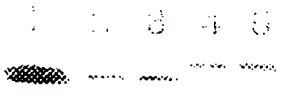


FIG. 11B.

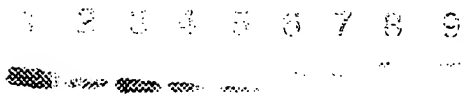


FIG. 11C.

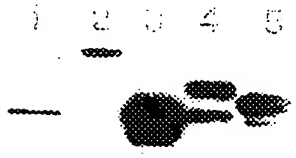


FIG. 11D.

DOCID: <WO___9821228A1_IA>

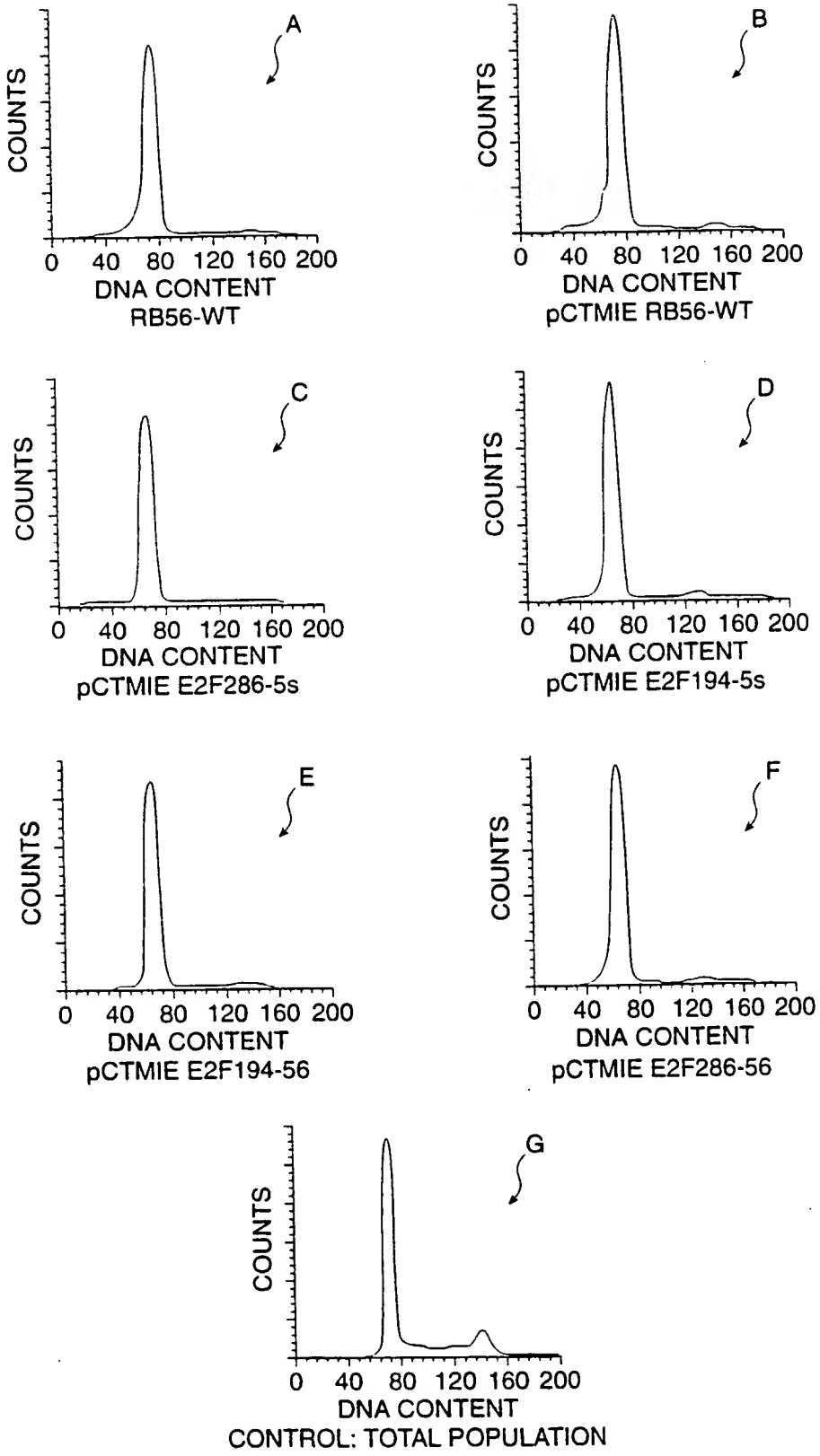


FIG. 12

SDOCID: <WO___9821228A1_IA>

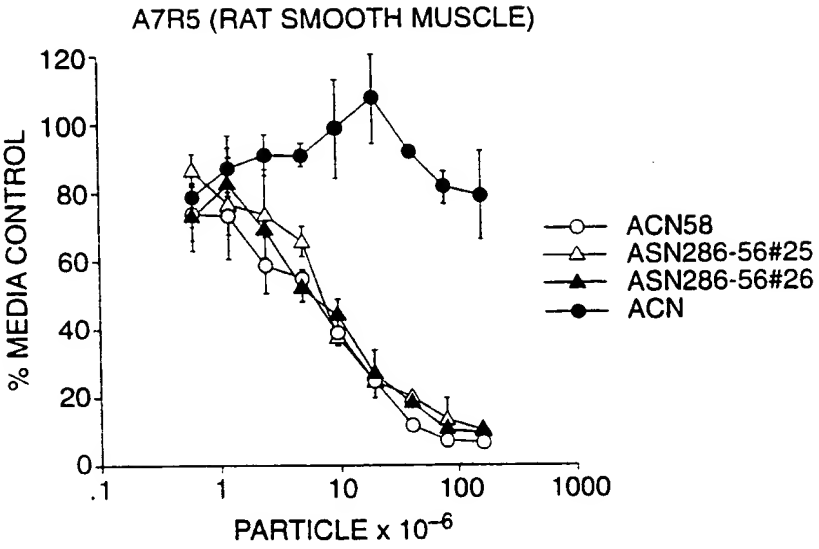


FIG. 13A

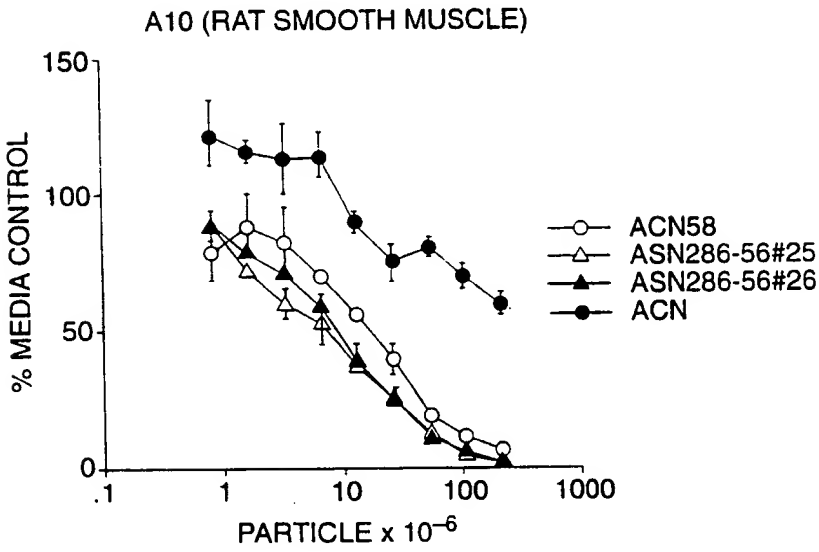


FIG. 13B

DOCID: <WO___9821228A1_IA>

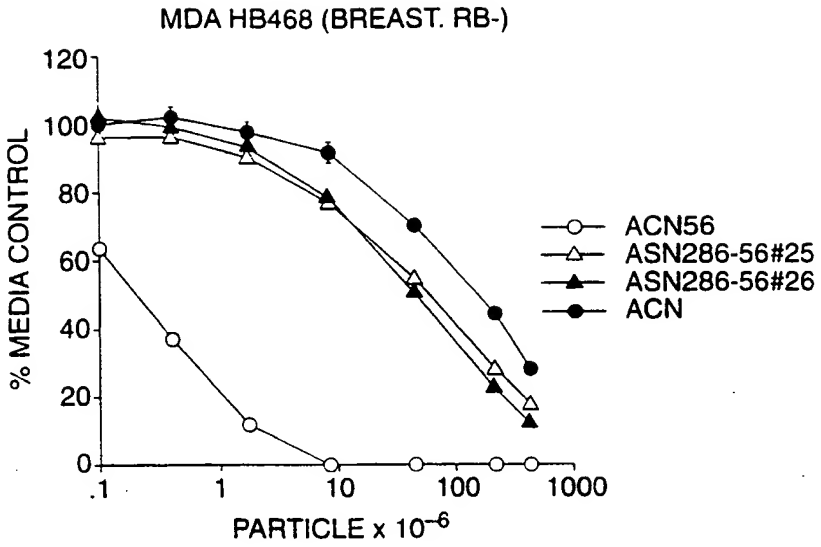


FIG. 14A

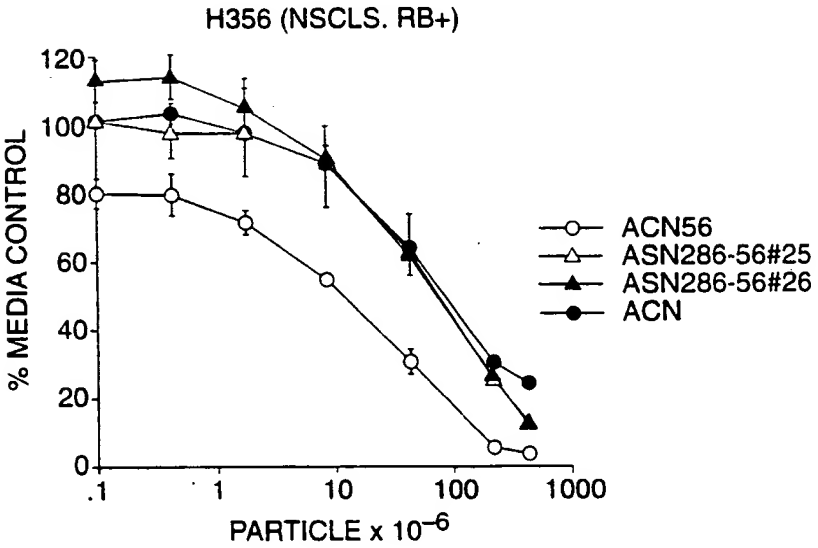
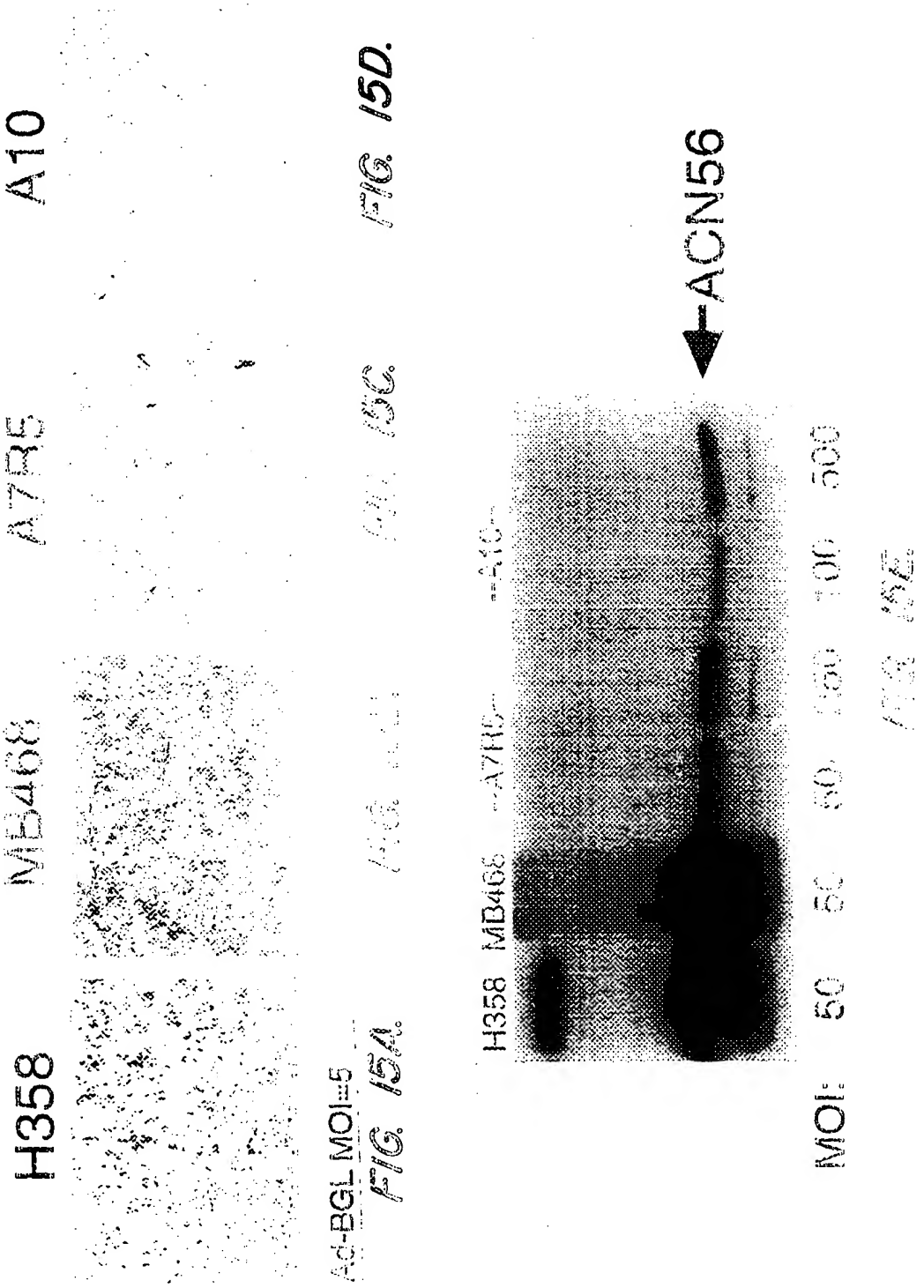


FIG. 14B

SDOCID: <WO___9821228A1_IA>



DOCID: <WO___9821228A1_IA>

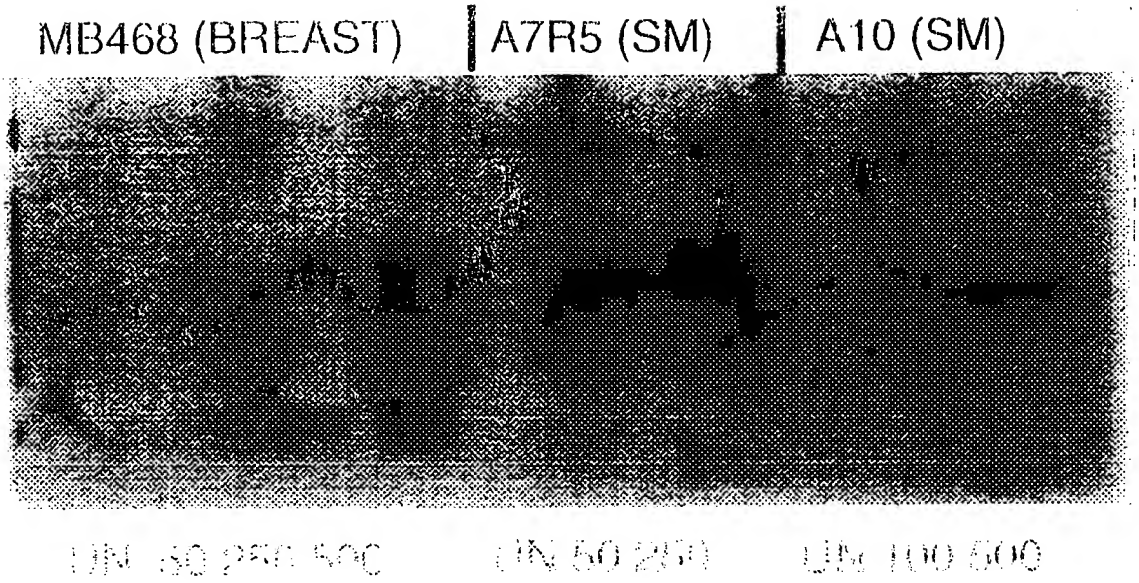


FIG. 16.

DOCID: <WO__9821228A1_IA>

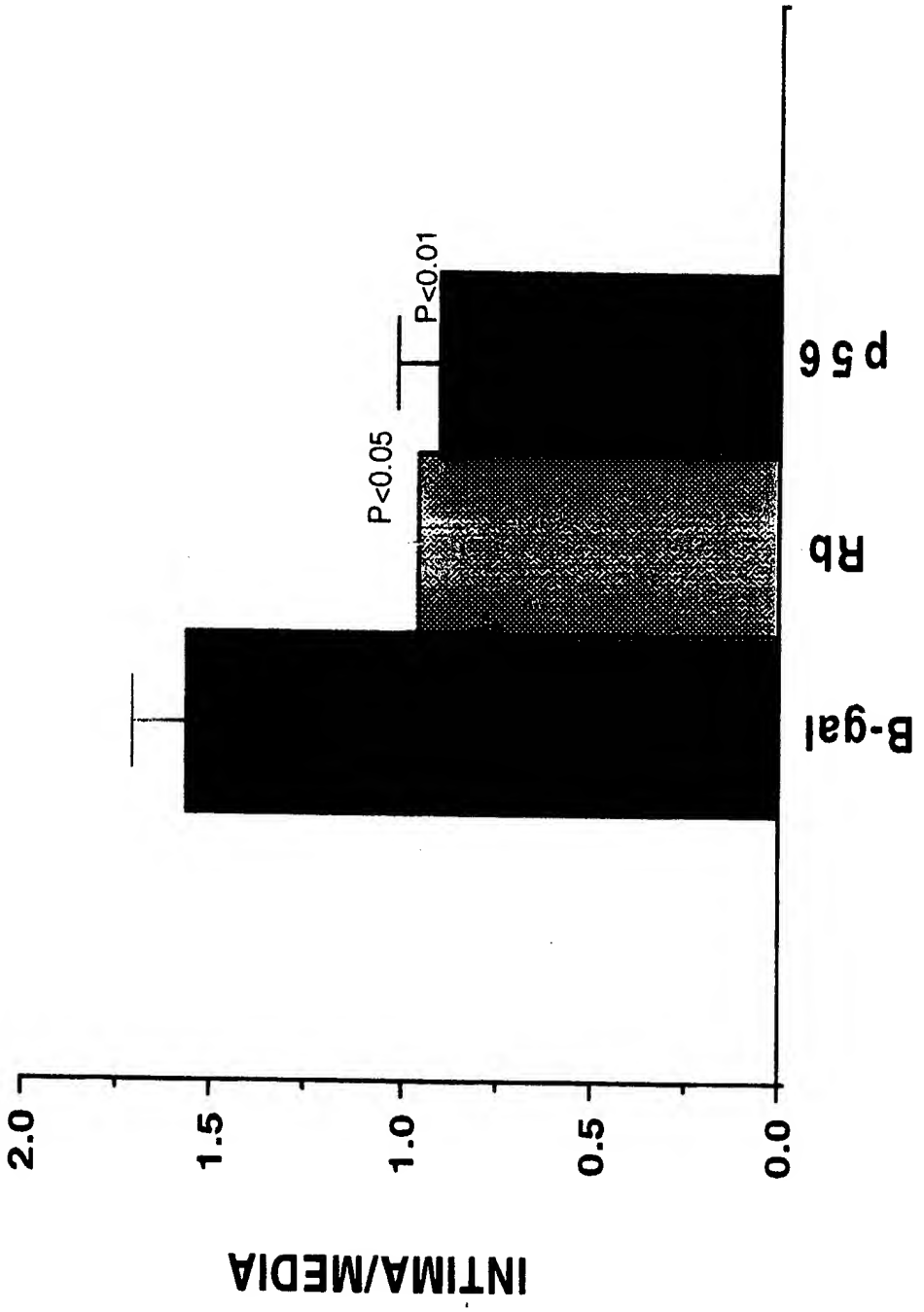


FIG. 17.

DOCID: <WO___9821228A1_IA>

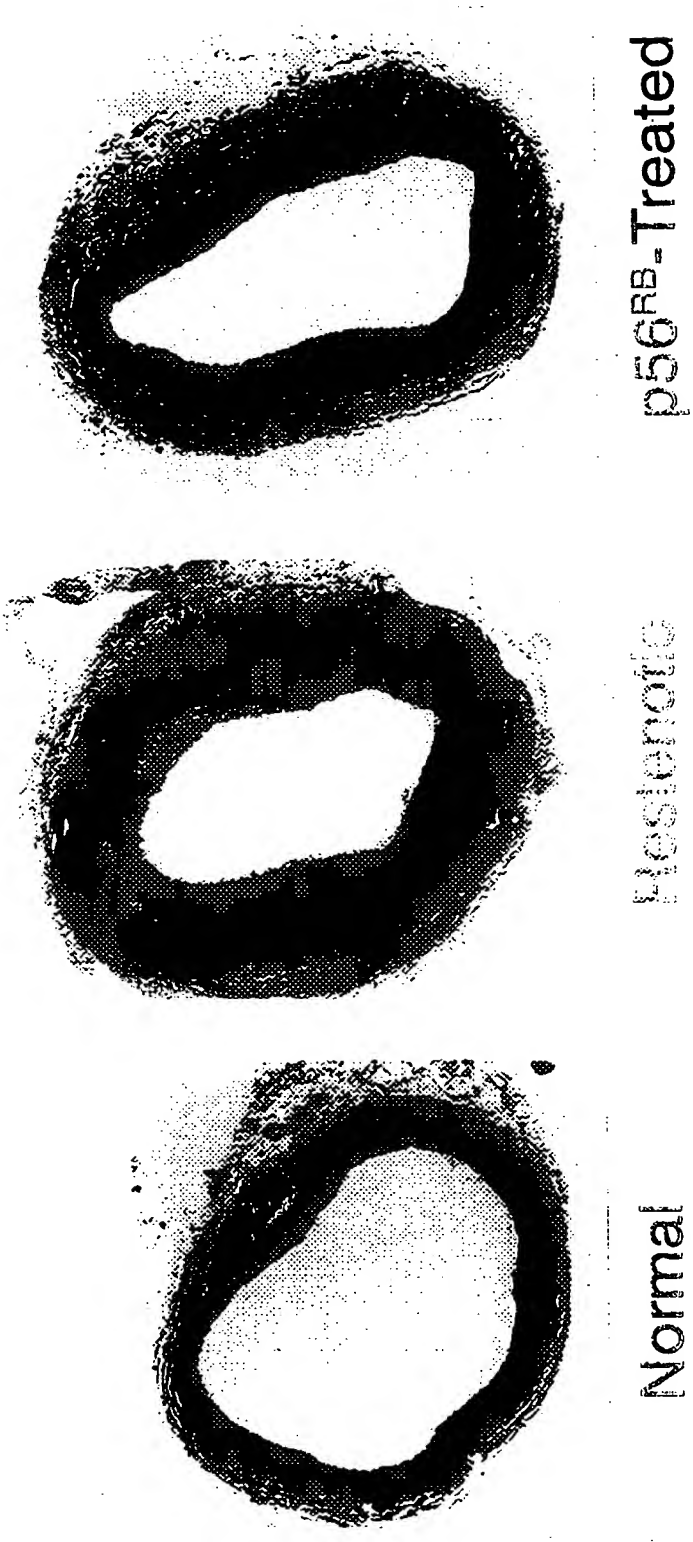
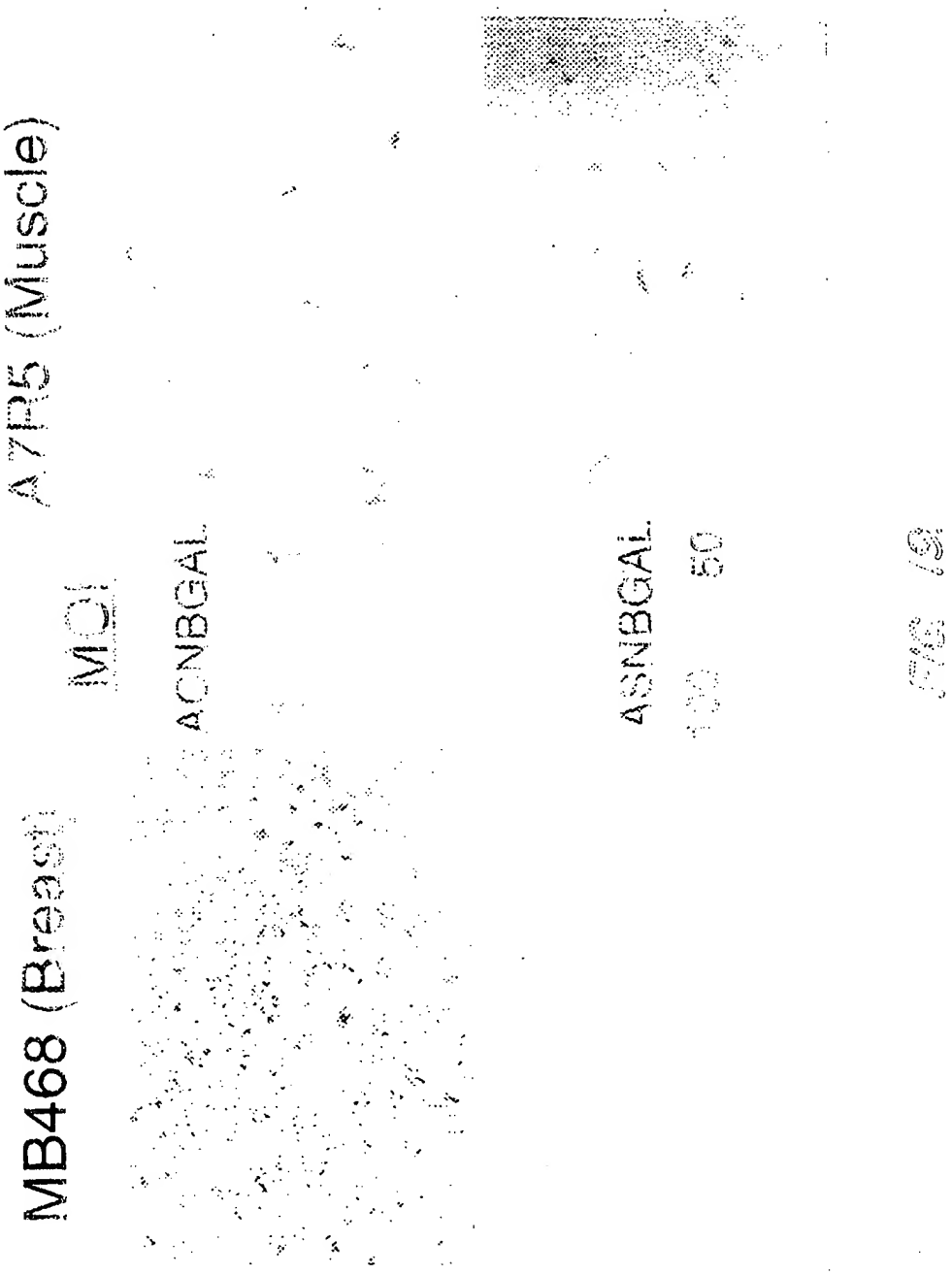


FIG 18

DOCID: <WO___9821228A1_IA>



DOCID: <WO___9821228A1_IA>

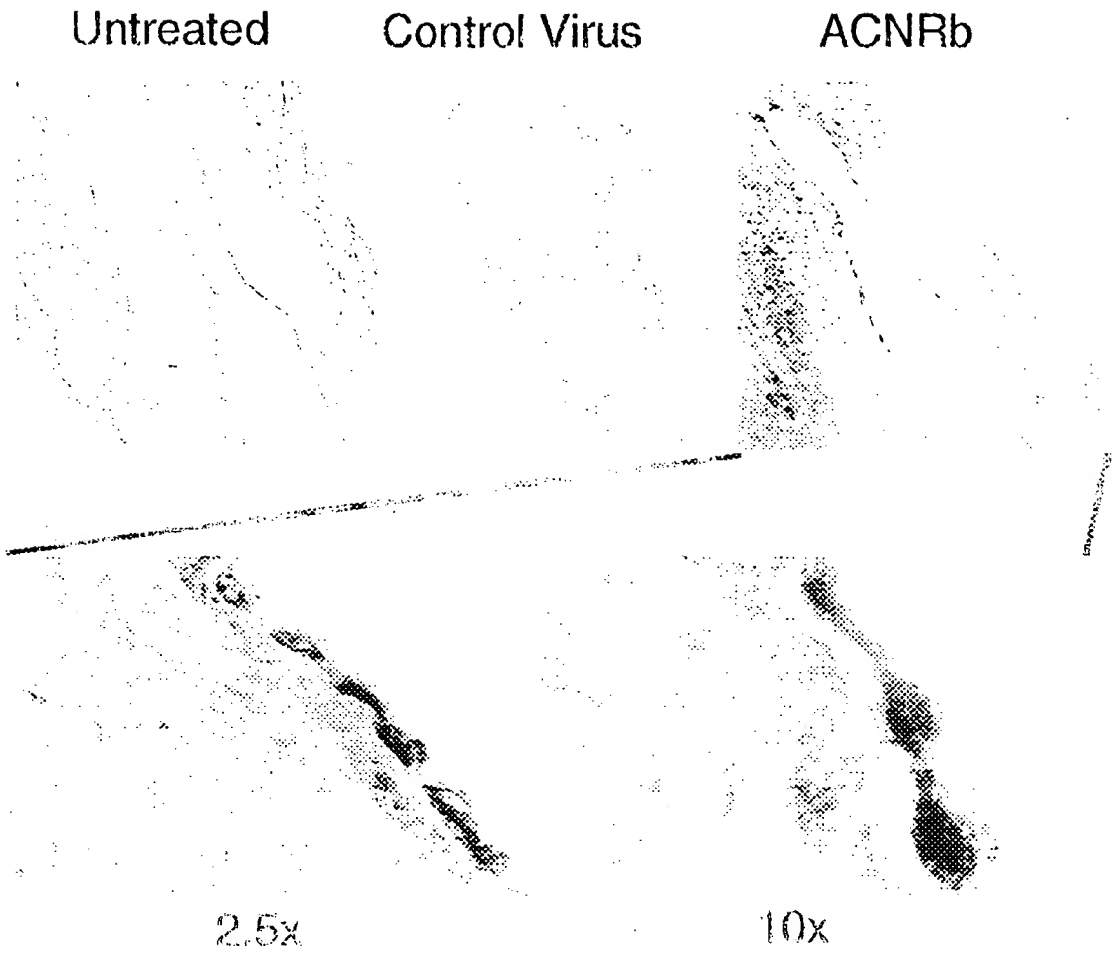


FIG. 20.

DOCID: <WO___9821228A1_IA>

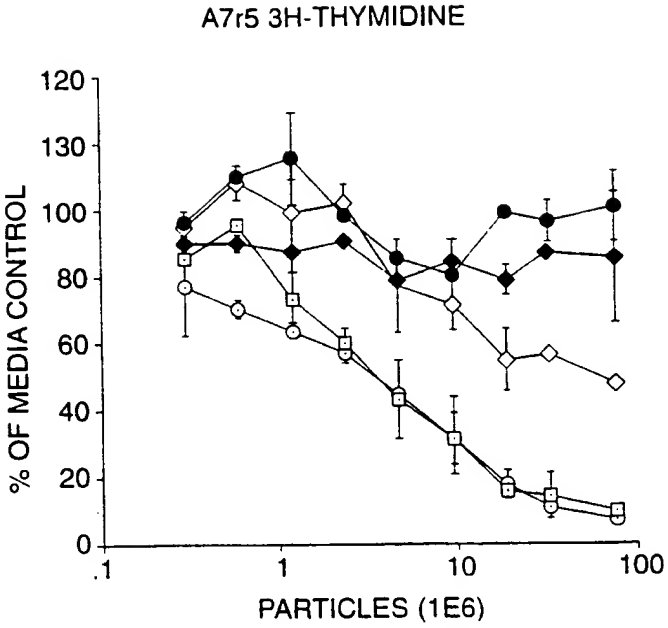


FIG. 21A

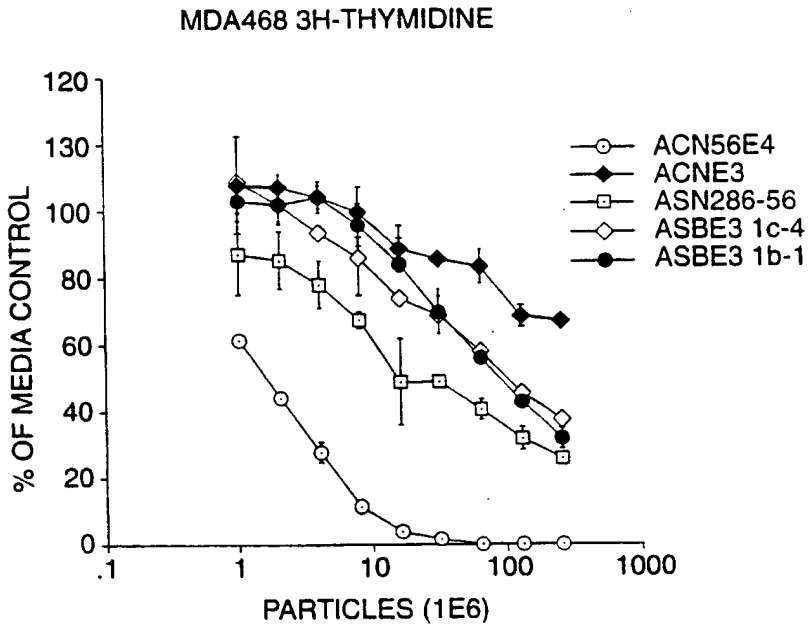


FIG. 21B

SDOCID: <WO___9821228A1_IA>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21821

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; CO7K 5/00; A61K 38/00, 35/12
US CL : 536/23.4, 24.5; 530/300; 424/277.1; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.4, 24.5; 530/300; 424/277.1; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, SCISEARCH, CANCERLIT, WPIDS, EMBASE
Search terms: retinoblastoma, RB polypeptide, adenovirus vector, transcription factor, restenosis, cancer treatment

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GOODRICH et al. Administration of a functional retinoblastoma polypeptide or protein-used to prevent and inhibit primary and secondary retinoblastoma linked cancers. WO 9507708 A2. 23 March 1995. Abstract.	1-36
Y	XU et al. Enhanced tumor suppressor gene therapy via replication-deficient adenovirus vectors expressing an N-terminal truncated retinoblastoma protein. Cancer Research. 15 May 1996. Vol.56. No.10. pages 2245-2249, especially abstract.	1-36

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 18 MARCH 1998	Date of mailing of the international search report 24 APR 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GEETHA P. BANSAL Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21821

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FUEYO et al. Expression of exogenousp16/CDKN2 produces growth arrest in a glioma cell line that does not express Rb protein. Proc. Annual Meeting American Association of Cancer Res. 1996. Vol 37. ppA49. Meeting Abstract.	1-36

DOCID: <WO___9821228A1_IA>